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Vitamin D and HIV Associated Tuberculosis

By

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DECLARATION

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Abstract

Background

Tuberculosis is a major cause of increased disease burden and death among HIV infected individuals in sub-Saharan Africa. In Europe vitamin D deficiency has been associated with increased risk of active tuberculosis. However, in subtropical Africa it has not been previously investigated.

Aims

The purpose of the study was to investigate association of vitamin D deficiency and tuberculosis in Cape Town; explore effects of smoking on vitamin D status; examine presence of seasonal variation of vitamin D levels and new tuberculosis case notifications. Determine the effects of vitamin D molecules, 25-hydroxycholecalciferol (25(OH)D₃) and 1, 25 dihydroxycholecalciferol (1,25 (OH)₂D₃) on HIV replication and *M. tuberculosis* growth and in addition investigate the influence of *M. tuberculosis* strain variation HIV replication.

Methods

25(OH)D₃ levels were measured by liquid chromatography mass spectrometry / mass spectrometry in a cross-sectional study of HIV uninfected (n=251) and HIV infected (n=229) individuals recruited from Ubuntu clinic, Khayelitsha and GF Jooste hospital. HIV replication and *M. tuberculosis* growth was monitored by measurement of p24 protein and colony forming units respectively in infected pro-inflammatory (MΦ1) and anti-inflammatory (MΦ2) macrophages.

Results

Vitamin D deficiency (25(OH)D₃ <50 nmol/L) was associated with susceptibility to active tuberculosis in HIV uninfected individuals (odds ratio = 5.2, 95% confidence interval: 2.8–9.7; p < 0.001) and HIV infected individuals (odds ratio = 5.6, 95% confidence interval: 2.7–11.6; p < 0.001) in comparison to latent tuberculosis infection (LTBI).

There was no independent association between smoking and vitamin D status. The mean 25(OH)D3 levels varied with season with lowest levels in July to September and maximum levels reached in January to March inclusive (30.7 vs. 56.8 nmol/L, respectively; $P < 0.001$). The quarterly new tuberculosis case notifications for the city of Cape Town were higher from October to December and lower in April to June (5,080 vs. 4,222; $P < 0.001$).

In M ϕ 1, 25(OH)D3 and 1,25(OH)₂D3 inhibited HIV replication after 144 hrs ($p < 0.05$ & $p < 0.001$ respectively) while 1,25(OH)₂D3 inhibited *M. tuberculosis* growth after 240 hrs of mono-infection ($p < 0.05$). During co-infection 25(OH)D3 and 1,25(OH)₂D3 restricted HIV replication after 72 hrs and *M. tuberculosis* growth after 168 hrs ($p < 0.05$ in all cases). In M ϕ 2, only 25(OH)D3 inhibited HIV replication after 240 hrs of mono-infection ($p < 0.05$) and vitamin D molecules had no significant effect on HIV replication and MTB growth during co-infection. *M. tuberculosis* strain variation had no significant effect on HIV replication in M ϕ 1 and M ϕ 2.

Conclusion

Vitamin D supplementation can be a possible safe way to enhance immunity against *M. tuberculosis* and HIV. There is need for a randomized clinical trial to explore the preventive effect of vitamin D on active tuberculosis in this high disease burden setting.

Table of Contents

DECLARATION	II
ABSTRACT	III
LIST OF FIGURES	VIII
LIST OF TABLES	X
ACKNOWLEDGEMENTS	XI
ABBREVIATIONS	XII
CHAPTER 1: GENERAL INTRODUCTION	1
1.1 Introduction	1
1.1.1 HIV structure and molecular biology	2
1.1.2 <i>Mycobacterium tuberculosis</i> biology	3
1.1.3 Vitamin D physiology and metabolism	5
1.2 Hypothesis	10
1.3 Aims of Study	10
1.4 Objectives	10
1.5 Overview of thesis	11
CHAPTER 2: MATERIALS AND METHODS	12
2.1 Study site and participants	12
2.1.1 Ethics approval	12
2.1.2 Recruitment and study design	12
2.1.3 Clinical data capturing	13
2.1.4 Screening tests	13
2.1.5 City of Cape Town tuberculosis notifications	13
2.1.6 Measurement of the 25-hydroxycholecalciferol (25(OH)D3)	13
2.1.7 Peripheral blood mononuclear cells (PBMC) preparation, positive selection of CD14+ cells and generation of monocyte-derived macrophages (MDM).	15
2.1.8 Flow cytometry analysis	16
2.1.9 Preparation of <i>M. tuberculosis</i> stock	18
2.1.10 Growth curves	19
2.1.11 Preparation of the HIV-1 BAL	20
2.1.12 Co-infection of MDM with HIV and <i>M. tuberculosis</i> strains	21
2.1.13 HIV/TB co-infection in presence of vitamin D molecules	22
2.1.14 Measurement of p24 in supernatants	23
2.1.15 Statistical analysis	23
2.2 Materials	24

CHAPTER 3: RECIPROCAL SEASONAL VARIATION IN VITAMIN D STATUS AND TUBERCULOSIS NOTIFICATIONS IN CAPE TOWN, SOUTH AFRICA.	27
3.1 Literature review	27
3.1.1 Vitamin D status	27
3.1.2 Vitamin D deficiency	28
3.1.3 Vitamin D and Tuberculosis	28
3.1.4 Vitamin D and HIV	32
3.2 Results	35
3.2.1 Characteristics of study participants	35
3.2.2 Other infections	37
3.2.3 Categorizing vitamin D status of study participants	38
3.2.4 Correlates of 25(OH)D3 Concentrations	42
3.2.5 Relationship between participant 25(OH)D3 levels and season	43
3.2.6 Correlation between serum 25(OH)D3 levels and CD4+ cells	45
3.3 Discussion	45
CHAPTER 4: EFFECT OF SMOKING ON VITAMIN D STATUS	52
4.1 Literature review	52
4.1.1 Effects of smoking on the immune system	52
4.1.2 Effects of smoking on bone and vitamin D	53
4.1.3 Vitamin D, smoking and respiratory disease	54
4.2 Results	55
4.2.1 Demographic and clinical characteristics of study participants	55
4.2.2 Vitamin D status of study participants	58
4.2.3 Analysis of risk factors of vitamin D status	60
4.3 Discussion	63
CHAPTER 5: IMMUNOPHENOTYPING OF MΦ-1 AND MΦ-2	67
5.1 Literature review	67
5.1.1 The mononuclear phagocyte system	67
5.1.2 Roles for macrophages in immunity	68
5.1.3 Macrophage polarization and activation	69
5.1.4 <i>In vitro</i> generation of macrophages	72
5.2 Results	75
5.2.1 Morphological characteristics of MΦ1 and MΦ2	75
5.2.2 Analysis of MDM by flow cytometry	76
5.3 Discussion	81
CHAPTER 6: MACROPHAGE PHENOTYPE DICTATES CELLULAR ABILITY TO CONTROL <i>M. TUBERCULOSIS</i> AND HIV-1 REPLICATION IN RESPONSE TO VITAMIN D	84
6.1 Literature review	84
6.1.1 Vitamin D and monocyte/macrophage biology	84
6.1.2 Vitamin D signalling and HIV-1 long terminal repeat (LTR)	85
6.1.3 Effects of vitamin D on HIV-1 and <i>M. tuberculosis</i>	87

6.2 Results	89
6.2.1 The effect of vitamin D on HIV-1 replication	89
6.2.2 The effect of vitamin D on HIV-1 replication during co-infection with <i>M. tuberculosis</i> .	91
6.2.3 The effect of vitamin D on Uptake of <i>M. tuberculosis</i>	92
6.2.4 The effect of vitamin D on the growth of <i>M. tuberculosis</i> .	93
6.2.5 The effect of macrophage phenotype on <i>M. tuberculosis</i> growth	96
6.3 Discussion	97
 CHAPTER 7: THE INFLUENCE OF MACROPHAGE PHENOTYPE ON THE DIFFERENTIAL GROWTH OF <i>M. TUBERCULOSIS</i> STRAINS AND HIV-1 REPLICATION DURING CO-INFECTION	 104
7.1 Literature review	104
7.1.1 Clinical outcomes associated with <i>M. tuberculosis</i> strain variation.	105
7.1.2 Immunopathogenesis of HIV and <i>M. tuberculosis</i> co-infection	107
7.2 Results	111
7.2.1 Comparison of growth kinetics of <i>M. tuberculosis</i> strains	111
7.2.2 The effects of <i>M. tuberculosis</i> strain on HIV replication	112
7.2.3 The effect of HIV on <i>M. tuberculosis</i> strains growth.	116
7.2 Discussion	123
 CHAPTER 8: GENERAL CONCLUSION	 127
8.1 Presentations and publications	129
8.1.1 Publications	129
8.1.2 Poster presentation	129
8.1.3 Poster abstracts	130
 BIBLIOGRAPHY	 131

List of Figures

FIGURE 1.1. NEIGHBOR-JOINING PHYLOGENY OF <i>M. TUBERCULOSIS</i> COMPLEX GENOME SEQUENCES.....	5
FIGURE 1.2 VITAMIN D METABOLISM.....	6
FIGURE 3.1 VITAMIN D AND THE MACROPHAGE ANTIMICROBIAL RESPONSE TO TB	33
FIGURE 3.2 SERUM 25(OH)D3 CONCENTRATION STRATIFIED BY HIV AND TB STATUS.	39
FIGURE 3.3 SEASONAL VARIATION IN MEAN SERUM 25(OH)D3 CONCENTRATION, ALL STUDY PARTICIPANTS.	44
FIGURE 3.4 NEW TB NOTIFICATIONS BY QUARTER, CITY OF CAPE TOWN, 2003 TO 2010.....	44
FIGURE 3.5 CORRELATION OF 25(OH)D3 LEVELS WITH CD4 AMONG THE HIV INFECTED PARTICIPANTS.....	45
FIGURE 3.6 SPECTRUM OF TUBERCULOSIS INFECTION WITH VITAMIN D	49
FIGURE 4.1 EFFECTS OF SMOKING IN LUNGS.	53
FIGURE 4.2 SERUM 25(OH)D3 BY STRATIFIED BY SMOKING STATUS. (A) DIVIDED INTO 3 GROUPS (B) DIVIDED INTO 2 GROUPS (EVER SMOKED COMPOSED OF CURRENT SMOKERS AND EX-SMOKERS).....	58
FIGURE 5.1 EFFECTS OF CSFs ON DIFFERENTIATION OF MONOCYTES TO MACROPHAGES (A) MΦ-1 (B) MΦ-2	76
FIGURE 5.2. THE GATING STRATEGY: MACROPHAGES WERE IDENTIFIED BY STAINING FOR CD11b. FIRST GATE WAS SET IN A FSC vs. SSC DOT PLOT (A) AND APPLIED TO A CD11b vs. SSC DOT PLOT (B) TO SET THE MACROPHAGE GATE AROUND THE CD11b POSITIVE CELLS (C).....	76
FIGURE 5.3 FLOW CYTOMETRY DOT PLOTS OF MΦ-1 AND MΦ-2 FROM A REPRESENTATIVE DONOR. CELLS WERE STAINED WITH LABELLED MONOCLONAL ANTIBODIES (A) FSC vs. SSC (B) CD33 vs. TLR2 (C) CCR5 vs. HLADR (D) CD33 vs. CD209 (E) CD14 vs. TLR2 (F) CD16 vs. CD15	77
FIGURE 5.4 EXPRESSION OF CELL SURFACE RECEPTORS ON MΦ-1 AND MΦ-2. THE OPEN HISTOGRAMS REPRESENT CELLS STAINED WITH SPECIFIC ANTIBODY AND THE SOLID HISTOGRAMS REPRESENT NEGATIVE CONTROLS.....	79
FIGURE 5.5 QUANTITATIVE COMPARISON OF PHENOTYPIC CHARACTERISTICS OF MΦ-1 AND MΦ-2.....	80
FIGURE 6.1 STRUCTURE OF THE HIV-1 5'-LTR OF HIV-1 SUBTYPE B	86
FIGURE 6.2. THE EFFECT OF 1,25(OH) ₂ D3 ON HIV-1 REPLICATION AFTER (A) 144 HRS AND (B) 240 HRS OF INFECTION.....	90
FIGURE 6.3. EFFECT OF 25(OH)D3 AND 1,25(OH) ₂ D3 ON HIV-1 REPLICATION 72 HRS AFTER COINFECTION WITH OR WITHOUT <i>M. TUBERCULOSIS</i> . (A) MΦ-1 AND (B) MΦ-2.	92
FIGURE 6.4. THE EFFECT OF VITAMIN D MOLECULES ON THE UPTAKE OF <i>M. TUBERCULOSIS</i> IN (A) MΦ-1 AND (B) MΦ-2 GROWN FOR 168 HRS IN EITHER THE PRESENCE OR ABSENCE OF HIV-1 INFECTION ..	93
FIGURE 6.5. GROWTH OF <i>M. TUBERCULOSIS</i> OVER 168 HRS IN THE PRESENCE OR ABSENCE OF VITAMIN D AND OR HIV-1 IN (A) MΦ-1 AND (B) MΦ-2.....	94
FIGURE 6.6. THE EFFECT OF VITMAIN D ON INTRACELLUAR <i>M. TUBERCULOSIS</i> (MTB) GROWTH AT 24 HRS (A-B) AND 96 HRS (C-D) POST- <i>M. TUBERCULOSIS</i> INFECTION OF MΦ-1 (A,C) AND MΦ-2 (B,D) IN THE PRESENCE (HIV+MTB) OR ABSENCE (MTB) OF HIV-1 INFECTION 168 HRS PRE- <i>M. TUBERCULOSIS</i> INFECTION.....	95
FIGURE 6.7. THE EFFECT OF VITAMIN D ON INTRACELLULAR <i>M. TUBERCULOSIS</i> (MTB) AFTER 168 HRS IN (A) MΦ-1 AND (B) MΦ-2 IN THE PRESENCE OR ABSENCE OF HIV-1 CO-INFECTION (HIV+MTB).....	96
FIGURE 7.1 GROWTH KINETICS OF <i>M. TUBERCULOSIS</i> STRAINS H37Rv, LAM3 AND HN878 IN MIDDLEBROOK 7H9 BROTH SUPPLEMENTED WITH ADC IN TRIPLICATES..	112
FIGURE 7.2 THE EFFECT OF <i>M. TUBERCULOSIS</i> STRAIN VARIATION ON HIV-1 p24 LEVELS AT SPECIFIC TIME POINTS (A) 4 HRS, (B) 24 HRS, (C) 96 HRS AND (D) 168 HRS.....	113
FIGURE 7.3 COMPARISON OF HIV REPLICATION IN MΦ-1 AND MΦ-2 AT SPECIFIC TIME POINTS (A) 4 HRS, (B) 24 HRS, (C) 96 HRS AND (D) 168 HRS.....	114
FIGURE 7.4 EFFECT OF <i>M. TUBERCULOSIS</i> STRAINS ON HIV REPLICATION IN MΦ-1 AND MΦ-1 MACROPHAGES AT SPECIFIC TIME POINTS (A) 4 HRS, (B) 24 HRS, (C) 96 HRS AND (D) 168 HRS..	115
FIGURE 7.5 THE GROWTHS CURVE OF HIV IN MACROPHAGES (A) REPRESENTS MΦ-1 AND (B) MΦ-2.....	116

FIGURE 7.6 TWO-WAY ANOVA OF UPTAKE OF <i>M. TUBERCULOSIS</i> STRAINS (A) HIV UNINFECTED (B) HIV INFECTED..	118
FIGURE 7.7 VARIATION IN GROWTH OF THE <i>M. TUBERCULOSIS</i> STRAINS H37Rv, LAM3 AND HN878 IN MΦ- 1 AND MΦ -2 AFTER (A) 4 HRS, (B) 24 HRS (C) 96HRS AND (D) 168HRS OF CULTURE, (N=5)..	119
FIGURE 7.8 THE GROWTH OF <i>M. TUBERCULOSIS</i> STRAINS IN MΦ-1 STRATIFIED BY HIV INFECTION STATUS AFTER (A) 24 HRS, (B) 96 HRS AND (C) 168 HRS.....	121
FIGURE 7.9 THE GROWTH OF <i>M. TUBERCULOSIS</i> STRAINS IN MΦ-2 STRATIFIED BY HIV INFECTION STATUS AFTER (A) 24 HRS, (B) 96 HRS AND (C) 168 HRS.....	121
FIGURE 7.10 GROWTH CURVE OF <i>M. TUBERCULOSIS</i> STRAINS IN (A) MΦ-1 AND (B) MΦ-2..	122

University of Cape Town

List of Tables

TABLE 2.1 ANTIBODY LIST	17
TABLE 2.2 FLOW CYTOMETER SET UP.....	18
TABLE 2.3 MATERIALS AND REAGENTS USED IN METHODS DESCRIBED ABOVE	24
TABLE 3.1 DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF HIV UN-INFECTED STUDY PARTICIPANTS.	36
TABLE 3.2 DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF HIV INFECTED STUDY PARTICIPANTS.	37
TABLE 3.3 NUMBER AND TYPE OF OPPORTUNISTIC INFECTIONS IN THE OI GROUP	38
TABLE 3.4 SERUM 25(OH)D3 CONCENTRATIONS OF HIV UNINFECTED STUDY PARTICIPANTS	40
TABLE 3.5 SERUM 25(OH)D3 CONCENTRATIONS OF HIV INFECTED STUDY PARTICIPANTS	41
TABLE 3.6 MEAN SERUM 25(OH)D3 OF HIV INFECTED AND UNINFECTED CLINICAL GROUPS.....	42
TABLE 3.7 CORRELATES OF VITAMIN D STATUS IN STUDY PARTICIPANTS: UNIVARIATE AND MULTIVARIATE ANALYSIS.....	43
TABLE 4.1 DEMOGRAPHIC AND CLINICAL CHARACTERISTICS OF STUDY PARTICIPANTS.	57
TABLE 4.2 SERUM 25(OH)D3 CONCENTRATIONS OF STUDY PARTICIPANTS STRATIFIED BY SMOKING STATUS.	59
TABLE 4.3 POST HOC ANALYSIS OF VITAMIN D DEFICIENCY AND MEAN VITAMIN D LEVELS OF SMOKING GROUPS.....	60
TABLE 4.4 CORRELATES OF VITAMIN D STATUS: UNIVARIATE AND MULTIVARIATE ANALYSIS.	61
TABLE 4.5 CORRELATES OF BMI: UNIVARIATE AND MULTIVARIATE ANALYSIS.....	62
TABLE 5.1 QUANTITATIVE COMPARISON OF THE MΦ-1 AND MΦ-2	78

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Abbreviations

1,25(OH) ₂ D ₃	1 α ,25-dihydroxycholecalciferol or calcitriol
25(OH)D ₃	25-hydroxycholecalciferol or calcidiol
7H9	Middlebrook 7H9 liquid media
ADC	albumin dextrose and catalase
OADC	oleic acid albumin dextrose and catalase
ANOVA	Analysis of variance
APC	Antigen presenting cell
ATB	Active tuberculosis
BCG	Bacille Calmette-Guérin
BMI	Body mass index
BSA	Bovine serum albumin
CD	Cluster of differentiation
CCL2	Chemokine (C-C motif) ligand 2
CCR5	C-C chemokine receptor 5
CFU	colony forming units
COPD	Chronic obstructive pulmonary disease
CXCL	Chemokine (C-X-C motif) ligand
CYP27B1	Cytochrome P450 27B1
CYP27A1	Cytochrome P450 27A1
DBP	Vitamin D-binding protein
dosR	dormancy survival regulon
EAST-6	6kDa early secretory antigenic target
EHR	Enduring hypoxic response
ELISA	Enzyme linked immunosorbent assay
FACS	Fluorescence-activated cell sorting
FCS	Fetal calf serum
g	centrifugal force
GM-CSF	Granulocyte/ Macrophage Colony Stimulating Factor
HIV	Human immunodeficiency virus
IFN- γ	Interferon gamma

IGRA	Interferon gamma release assay
I κ B	Inhibitor- κ B
IKK	I κ B kinase
IL-6	Interleukin 6
IQR	Inter quartile range
kDa	kiloDalton
LPS	lipopolysaccharide
LTBI	latent tuberculosis
LTR	long terminal repeat
MACS	magnetic cell sorting system
M-CSF	Macrophage-colony stimulating factor
MDM	monocyte derived macrophages
MDR	Multi drug resistant
M ϕ	macrophage
M ϕ 1	pro-inflammatory macrophages
M ϕ 2	anti-inflammatory macrophages
mg	milligram
MHC	Major histocompatibility complex
ml	milli liter
mm	milli meter
MOI	multiplicity of infection
MTB	<i>Mycobacterium tuberculosis</i>
ng	nanogram
NOD	Nucleotide oligomerization domain receptors
OD	optical density
OI	diseases other than tuberculosis
OR	odds ratio
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PE/PPE	group of gene family with the presence of N-terminal ProGlu/ProProGlu motifs
pg	picogram

PHA	phytohemagglutinin
PI3K	Phosphatidylinositol 3-kinase
PPD	Purified protein derivative
PTH	Parathyroid hormone
RD	region of difference
REC	Research ethics committee
ROS	reactive oxygen species
RNA	Ribonucleic acid
RNI	reactive nitrogen intermediates
rpm	rotations per minute
SSC	side scatter
SD	standard deviation
T-cell	T lymphocytes
TB	tuberculosis
TLR	Toll like receptor
Th1	Type 1 helper T cell
Th2	Type 2 helper T cell
TNF	Tumor necrosis factor
TST	Tuberculin skin test
UPLCMS/MS	Ultraperformance liquid chromatography mass spectrophotometry/ mass spectrophotometry
UVB	ultraviolet beta radiation
UVR	ultraviolet radiation
UV	ultraviolet
VDRE	Vitamin D response element
VDR	Vitamin D receptor
WHO	World Health Organization
XDR	Extensively drug resistance
μ	micro

Chapter 1: General Introduction

1.1 Introduction

Tuberculosis (TB) is a curable disease with the highest worldwide mortality after malaria. It kills one person every second which translate to approximately 4700 people everyday, one in four of these deaths occur in Human Immunodeficiency Virus (HIV) infected persons according to the latest World Health Organisation (WHO) statistics (WHO, 2011). Africa has the highest burden of TB/HIV co-infection with 82% of the world's cases.

According to WHO statistics, (2011) South Africa is one of the top five countries with the highest number of incident TB cases; 490 000 (0.4–0.59 million) (WHO, 2011).

The average incidence of tuberculosis in South Africa for 2010 was 981 cases/100 000 and in informal settlements such as Khayelitsha Township where 70% of adults presenting with TB are co-infected with HIV is 1600 cases/ 100 000 (WHO, 2011, Garone *et al.*, 2011). Consequently the combined burden and effect of TB and HIV/AIDS is immense particularly in Cape Town informal settlements, which is aggravated by increased rates of *M. tuberculosis* re-infection and multidrug resistant tuberculosis in HIV infected persons (Cox *et al.*, 2010, Gupta *et al.*, 2012). This demands more research into understanding of the interaction between *M. tuberculosis* and host in order to find better methods for the prevention, control and treatment of *M. tuberculosis* infection.

During co-infection it has been observed that HIV-1 predisposes individuals to activation of latent tuberculosis and progression of disease on exposure whereas *M. tuberculosis* increases progression of HIV infection towards development of AIDS. The major causes of reactivation of *M. tuberculosis* are not yet fully understood, low CD4 count, immunosuppression due to: AIDS, corticosteroid therapy, anti-TNF- α therapy and rare genetic defects in IL-12 and IFN- γ have been shown to result in increased risk of active tuberculosis but they do not contribute much to the overall global burden (Corbett *et al.*, 2003, Leandro *et al.*, 2009, Keane *et al.*, 2001). There is need for effective treatment and interventions, which will reduce the occurrence of

TB in HIV infected patients. Drug resistant TB is more common in people living with HIV/AIDS (Gandhi *et al.*, 2010) and the increased incidence of TB in HIV infected individuals has the effect of increasing the tuberculosis burden and transmission in the community. In recent years there are increasing reports on the importance of vitamin D in enhancing immunity against *M. tuberculosis* infection and improved outcome in HIV infected individuals (Khoo *et al.*, 2012).

1.1.1 HIV structure and molecular biology

HIV is a double stranded ribonucleic nucleic acid (RNA) retrovirus composed of a glycoprotein spiked membrane envelope generated from the host cell. The viral envelope is composed of host proteins and viral proteins gp120 and gp41, which are used for entry into target cells by binding to CD4 receptor in conjunction with the co-receptors C-C chemokine receptor 5 (CCR5) or chemokine (C-X-C) receptor 4 (CXCR4). On entry the nucleocapsid is dismantled and viral ribonucleic acid (RNA) is transcribed into complementary deoxyribonucleic acid (DNA) by the reverse transcriptase that is integrated into host DNA by the integrase enzyme (Krogstad, 2003).

The virus can remain in host cell latently and activation of the host cell stimulates viral DNA transcription followed by generation of viral proteins. HIV genome is composed of 9 kilobase pairs carrying 9 genes gag, polymerase (pol), envelope protein (env), viral infectivity factor (vif), viral protein U (vpu), transcriptional activator (tat), viral protein R (vpr), negative effector (nef) and regulator of viral gene expression (rev) genes that are flanked by the long terminal repeats on the 5' and 3' ends (Krogstad, 2003, Turner and Summers, 1999).

1.1.2 *Mycobacterium tuberculosis* biology

Infection with *Mycobacterium tuberculosis* occurs through the respiratory tract and inhalation of 1-5 bacilli can result in infection. The innate immune cells such as epithelial cells, alveolar macrophages and neutrophils are involved in early control of *M. tuberculosis* (Rivas-Santiago *et al.*, 2008). Detection of inhaled *M. tuberculosis* by alveolar macrophage's complement receptor 3, mannose receptors, scavenger receptors, nucleotide oligomerization domain like (NOD-like) receptors and Toll like receptors (TLR) stimulates its phagocytosis and secretion of pro-inflammatory cytokines; tumour necrosis factor alpha (TNF- α), interleukin 6 (IL-6), IL-1 β , interferon gamma (IFN- γ) and chemokines: chemokine (C-C motif) ligand 2 (CCL2), CCL3, CCL7, Chemokine (C-X-C motif) ligand 2 (CXCL2) and CXCL10 (Ferrero *et al.*, 2003). Fusion of the *M. tuberculosis* phagosome with lysosomes results in its destruction by reactive oxygen species (ROS), reactive nitrogen intermediates (RNI) and antimicrobial peptides (Sundaramurthy and Pieters, 2007). However in most cases *M. tuberculosis* inhibits fusion of its phagosome with lysosomes (Hmama *et al.*, 2004). This allows the bacilli to replicate inside the macrophages and be transported to other tissues.

The local inflammatory response generated by the macrophages and neutrophils results in the recruitment of T cells to the site of infection which walls off the infection foci resulting in granuloma formation (Houben *et al.*, 2006). Granulomas are composed of the bacilli in a caseous phase at the centre surrounded by layers of macrophages, epithelial cells and T cells. *M. tuberculosis* goes into latency a dynamic state where the bacilli activate the dormancy survival regulator (DosR) regulated and enduring hypoxic response (EHR) genes. It replicates at a very slow rate and is kept at bay by the macrophages and T cells that are constantly mounting an immune response against it and the immune cells in the granuloma are constantly being renewed (Barry *et al.*, 2009). The infection can remain controlled (latent) for many years or it can be activated into active infection by immunodeficiency caused by conditions such as HIV infection or malnutrition (Russell, 2001, Martineau *et al.*, 2007a).

1.1.2.1 *Mycobacterium tuberculosis* evolution and genetic variation

Mycobacterium tuberculosis belongs to the genus *Mycobacteria*, which is composed of many strains and most of which are non-pathogenic. *M. tuberculosis* is thought to have originated from Africa and then spread to the rest of the world (Gagneux and Small, 2007). *M. tuberculosis* strains have a high degree of evolutionary conservancy with similar DNA sequences and identical 16s RNA gene sequences with the other strains in the *Mycobacterium tuberculosis* complex (Brosch *et al.*, 2002).

The *M. tuberculosis* specific deletion 1 (TbD 1) is used to classify *M. tuberculosis* into “ancient” and “modern” strains, where the “modern” strains lack the TbD1 (Brosch *et al.*, 2002). Various molecular techniques are used to study the *M. tuberculosis* lineages and strains these include: large sequence polymorphism (LSP), single nucleotide polymorphism (SNP), restriction fragment length polymorphism of the IS6110, spoligotyping to identify unique spacers with clustered regulatory short palindromic repeats (CRISPR) or direct repeat region (DR) and the identification of variable number of tandem repeats -mycobacterial interspersed repetitive units (MIRU-VNTR) (Mathema *et al.*, 2006). Based on the LSP molecular method *M. tuberculosis* strains are divided into six phylogeographical lineages (Figure 1.1) (Gagneux and Small, 2007). The genetic variability of clinical strains of *M. tuberculosis* can result in phenotypic differences like transmissibility, virulence and pathogenicity that can have an impact on the clinical outcome of disease (Nicol and Wilkinson, 2008).

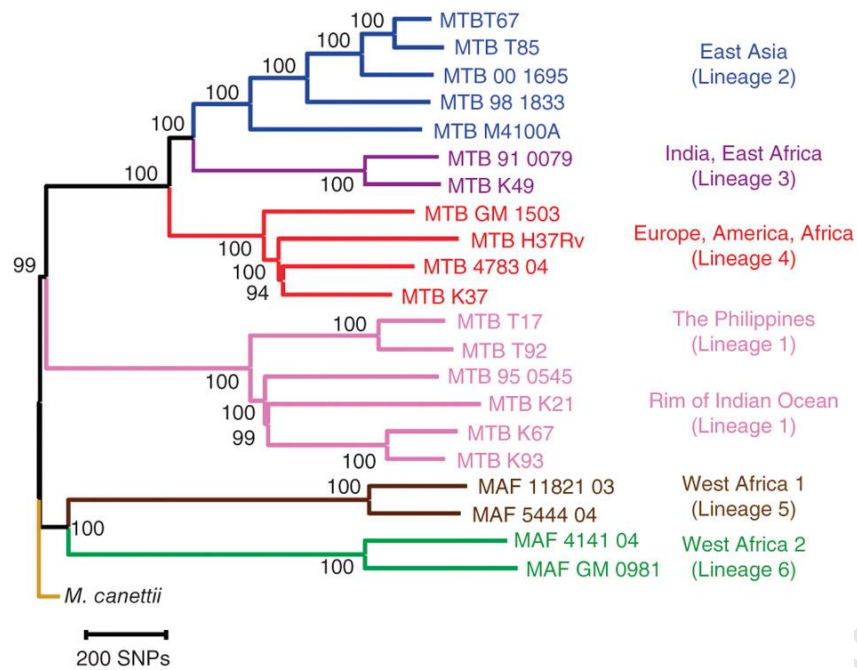


Figure 1.1. Neighbor-joining phylogeny of *M. tuberculosis* complex genome sequences (Comas *et al.*, 2010).

1.1.3 Vitamin D physiology and metabolism

Vitamin D has important biological and physiological functions in the body. There are 2 main types: cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2) (Figure 1.2). Vitamin D3 is obtained by use of ultraviolet B radiation (UVB) photosynthesis in the skin and through ingestion from the diet. Exposure to UVB in sunlight with wavelength 290-315 nm results in synthesis of vitamin D3 from 7-dehydrocholesterol (7-DHC) in the epidermis (Holick, 2007).

The skin is able to synthesize up to 3 000 IU of vitamin D3 when exposed to UVB for 5-10 minutes and it is self-limiting as the same UV starts to break it down when in excess (Holick, 2007). Continual exposure to ultraviolet radiation (UVR) results in the pre-vitamin D3 intermediates being converted to non-toxic lumisterol and tachysterol, which can be reversibly converted back to pre-vitamin D3 when concentrations are decreasing.

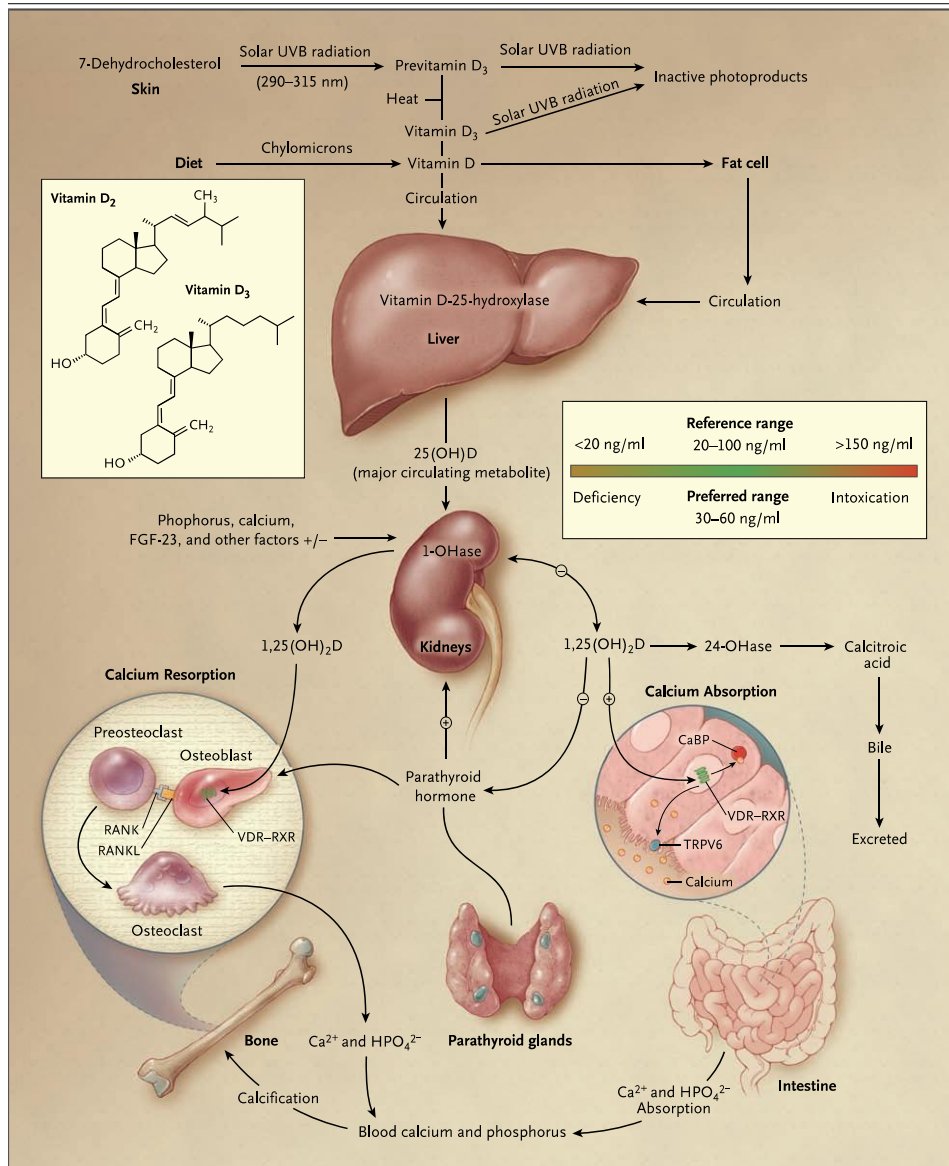


Figure 1.2 Vitamin D metabolism (Holick, 2007).

Dietary sources of vitamin D₂ include yeasts, fungi and mushrooms, whereas vitamin D₃ comes from cod liver oil, oily fish, eggs, salmon, and mackerel. Vitamin D₃ can be made synthetically from lanolin extracted from sheep wool fat through purification and extraction (Holick, 2007). The dietary sources of vitamin D are quite expensive. Although sunlight remains the cheapest source, it is not fully utilised because of changes in lifestyles. Fortification of foods and taking supplements are the possible cost effective options available.

Factors which affect vitamin D synthesis from sunlight are latitude, skin pigmentation, amount of clothing, time of day, season, diet, age, obesity, malabsorption, sunscreen use, liver and kidney disease. Melanin absorbs UVB in sunlight needed for vitamin D synthesis in the epidermis and in black Africans melanin has been associated with decreased vitamin D synthesis (Clemens *et al.*, 1982).

Dietary vitamin D3 or D2 is absorbed in the small intestines and is transported into circulation bound to vitamin D binding proteins and albumin. In the liver it is 25-hydroxylated to 25 hydroxycholecalciferol (25(OH)D3) by the microsomal and mitochondrial 25 hydroxylase enzymes such as CYP27A1 and CYP2R1. The 25(OH)D3 is transported to kidneys where it is hydroxylated to 1,25 dihydroxycholecalciferol (1,25(OH)₂D3) which is the active hormone (Holick, 2007). 1,25(OH)₂D3 synthesis is not only limited to kidneys, paracrine synthesis occurs in many extra-renal tissues which express CYP27B1: immune cells, brain tissue, osteoblasts, testis, pancreas, eyes and colon tissues (Hewison *et al.*, 2007).

Vitamin D actions are mediated via the vitamin D receptor (VDR) and through non-genomic and genomic signalling. In non-genomic signalling 1,25(OH)₂D3 binds to the membrane VDR which activates rapid signalling transduction pathways that induce intracellular physiological processes such as secretion of insulin, absorption of calcium, exocytosis and opening of voltage gated calcium, chloride channels and production of ROS (Norman *et al.*, 2004). The non-genomic effects take seconds to hours to occur whereas as those for genomic signalling take hours to days for their effects to manifest. Genomic signalling involves the binding of 1,25(OH)₂D3 to nuclear VDR which heterodimerises with retinoid X receptors resulting inactivation or suppression of target genes in the presence of co activators or co-repressor molecules (Rachez and Freedman, 2000).

The main classic functions of kidney produced 1,25(OH)₂D3 are bone growth and mineral metabolism (Figure 1.2). 1,25(OH)₂D3 synthesis increases the adsorption of calcium, phosphorous and magnesium in the intestines and kidneys.

Vitamin D deficiency causes inadequate calcification of bones, resulting in rickets, osteoporosis and fractures (Holick, 2007). Extra renally synthesized $1,25(\text{OH})_2\text{D}_3$ has autocrine, paracrine and intracrine functions in different tissues and cells. It controls many genes, some of which are involved in angiogenesis, apoptosis, cell proliferation and differentiation. $1,25(\text{OH})_2\text{D}_3$ has been implicated as important for a number of immunomodulatory activities on innate and adaptive immune system, such as antimicrobial activities against infectious diseases like tuberculosis and influenza. In non-communicable diseases, $1,25(\text{OH})_2\text{D}_3$ has been reported to mediate activities against cancer, cardiovascular disease, autoimmune disorders, hypertension, and diabetes mellitus (Heaney, 2008).

In the pre-antibiotic era vitamin D was used for the treatment of tuberculosis through sunbathing in sanatoriums, use of UV light lamps and consumption of cod liver oil. There is a high prevalence of vitamin D deficiency in different populations worldwide. A large amount of literature points towards a role of vitamin D in the control of HIV and drug sensitive and resistant tuberculosis infection (Martineau *et al.*, 2007a, Mehta *et al.*, 2011, Rathored *et al.*, 2012), through *in vivo*, *ex vivo* and *in vitro* studies that demonstrated the immunomodulatory effects of vitamin D (Martineau *et al.*, 2007c, Coussens *et al.*, 2012, Fabri *et al.*, 2011, Campbell and Spector, 2012). Therefore vitamin D can possibly be used as adjunctive immunotherapy against *M. tuberculosis*. Vitamin D deficiency is common among HIV infected individuals and it has been associated with increased disease progression (Mehta *et al.*, 2011).

However, there have been no studies to evaluate prevalence of vitamin D deficiency among tuberculosis and HIV infected persons in Cape Town, South Africa. Cape Town townships have some of the highest prevalence and incidence of tuberculosis due to biological, social and economic problems. The need to evaluate the prevalence of vitamin D deficiency in these high burden settings is great considering the possible role of vitamin D to immunity to HIV and *M. tuberculosis* infections.

The high prevalence of tuberculosis in Cape Town might potentially be related to vitamin D deficiency because Pettifor et al demonstrated that *in vitro* synthesis of vitamin D3 from 7-dehydrocholesterol was low in winter, it was one third of that in Johannesburg (Pettifor *et al.*, 1996).

Furthermore use of nucleoside reverse transcriptase and protease inhibitors of HIV have been shown to have adverse effects on vitamin D metabolism as well as rifampicin and isoniazid (Cozzolino *et al.*, 2003, Bout-Van Den Beukel *et al.*, 2008, Brodie *et al.*, 1982). Hence all these factors support the need for determination of vitamin D levels in this population. Previously smoking has been associated with vitamin D deficiency and Cape Town has the highest number of smokers in South Africa (Brot *et al.*, 1999, Chopra M *et al.*, 2007). Therefore investigation of the effect of smoking on the vitamin D levels of the study participants was also carried out.

Vitamin D can be used for targeted or mass supplementation as *in vivo* studies have shown it to be safe in HIV infected and un-infected individuals with latent and active tuberculosis (Arpadi *et al.*, 2009, Martineau *et al.*, 2009b). However there are conflicting reports on the effect of vitamin D on HIV replication. In light of *in vitro* studies which report increased HIV replication by vitamin D there is need to investigate the safety and effectiveness of vitamin D supplementation in HIV infected individuals. Therefore the study investigated the effect of vitamin D molecules 25(OH)D3 and 1,25(OH)₂D3 on HIV replication in macrophages.

Macrophages are the major target of *M. tuberculosis* infection as well as being reservoirs of HIV infection. *In vivo* macrophages occur as a spectrum of activation phenotypes depending on their microenvironment in the body. Therefore it is necessary to carry out the experiments in the two extremes of macrophage activation pro-inflammatory (MΦ1) and anti-inflammatory (MΦ2) macrophages (Verreck *et al.*, 2004). Moreover in light of recent reports of differential immune responses as a result of genetic variation of *M. tuberculosis* strains in peripheral blood mononuclear cells (Ranjbar *et al.*, 2009a), I sought to further investigate the role of different *M. tuberculosis* strains on HIV replication in macrophages.

1.2 Hypothesis

1. Vitamin D deficiency is associated with tuberculosis in Cape Town, South Africa.
2. Vitamin D deficiency is associated with smoking.
3. Vitamin D does not enhance HIV replication.
4. HIV replication is differentially influenced by *M. tuberculosis* strain variation.

1.3 Aims of Study

1. The primary goal of the thesis was to determine the prevalence of vitamin D deficiency in HIV infected and uninfected patients with tuberculosis in Cape Town, South Africa.
2. To investigate the relationship between seasonal variation of vitamin D levels of study participants and new tuberculosis case notifications of the city of Cape Town from 2003 to 2010.
3. To investigate the influence of vitamin D molecules and *M. tuberculosis* strain variation on HIV replication and *M. tuberculosis* growth.

1.4 Objectives

1. To determine total 25 hydroxycholecalciferol levels in patients with *M. tuberculosis* and appropriate controls.
2. To examine the 25(OH)D₃ levels of smokers, ex-smokers and non-smokers.
3. To investigate the effect of 25(OH)D₃ and 1,25(OH)₂D₃ on HIV replication in GM-CSF macrophages (M ϕ 1) and M-CSF macrophages (M ϕ 2).
4. To establish the effect of 25(OH)D₃ and 1,25(OH)₂D₃ on *M. tuberculosis* growth in M ϕ 1 and M ϕ 2.
5. To investigate the effect of *M. tuberculosis* strains H37Rv, LAM3 and HN878 on HIV replication in M ϕ 1 and M ϕ 2.
6. To investigate the effect of HIV on growth of *M. tuberculosis* strains in M ϕ 1 and M ϕ 2.

1.5 Overview of thesis

Chapter 1 is a general introduction to the thesis.

Chapter 2 describes the materials and methods that were used in the investigations.

Chapter 3 describes the investigation of the association of vitamin D deficiency and Tuberculosis in Cape Town South Africa.

Chapter 4 examines the effect of smoking on vitamin D levels.

Chapter 5 describes the immunophenotype of the M ϕ 1 and M ϕ 2 used in the HIV and *M. tuberculosis* co-infection model.

Chapter 6 describes the *in vitro* effects of vitamin D molecules on HIV replication and *M. tuberculosis* growth in M ϕ 1 and M ϕ 2.

Chapter 7 describes the reciprocal effect of *M. tuberculosis* strains and HIV on each other's growth during co-infection in M ϕ 1 and M ϕ 2.

Chapter 8 describes the overall conclusions of this thesis.

Chapter 2: Materials and Methods

2.1 Study site and participants

2.1.1 Ethics approval

Ethical approval for the studies was obtained from the University of Cape Town Ethics committee:

1. Cross sectional study for vitamin D and tuberculosis in Cape Town REC REF 030/2010.
2. Recruitment and collection of blood from healthy normal donors for *in vitro* studies REC REF 261/2008.

2.1.2 Recruitment and study design

The study design used for the Vitamin D and tuberculosis study was cross sectional. Participants were recruited from the Ubuntu clinic at Khayelitsha site B and the GF Jooste hospital in Mannenberg, Cape Town, between April 2005 and January 2010. The study recruited 480 participants that were composed of 208 males and 272 females. The inclusion criterion included HIV infected and un-infected adults over the age of 18 years and met the clinical criteria as detailed below. Patients were recruited before initiation of either anti mycobacterial or highly active antiretroviral therapy. The nature of the study procedures, potential risks, benefits of the study, maintenance of confidentiality and voluntary nature of participation was explained to all the study participants before written informed consent was obtained. The individuals who met the full clinical criteria for their final study group were included in the final data analysis.

2.1.3 Clinical data capturing

The participants were screened for common symptoms of tuberculosis such as cough, weight loss, fever, loss of appetite and night sweats. Clinical and demographic data which was captured included HIV status, age, history of TB, BCG scar, CD4 cell count, gender, body mass index (BMI), employment status, smoking status, level of education and presence of any other infection or disease other than *M. tuberculosis*.

2.1.4 Screening tests

Blood samples were collected into heparin anti-coagulated and plain tubes from the study participants by venepuncture. HIV was diagnosed at the clinic using the Determine HIV 1/2 test (Abbott) and the confirmatory enzyme linked immunosorbent assay (ELISA) was performed at the reference National Health Laboratory Service (NHLS). Sputum samples were collected and sent to the NHLS where *M. tuberculosis* infection was diagnosed by smear microscopy or culture in the mycobacterial growth indicator tube (MGIT) system. Latent tuberculosis was diagnosed in individuals with no clinical symptoms of tuberculosis and negative sputum smear and culture for *M. tuberculosis* by a positive tuberculin test (TST) and in-house interferon gamma release assay (IGRA) (Oni *et al.*, 2012).

2.1.5 City of Cape Town tuberculosis notifications

The new tuberculosis case notifications for the City of Cape Town for the period 2003 to 2010 inclusive were obtained from the City Department of Health database.

2.1.6 Measurement of the 25-hydroxylcholecalciferol (25(OH)D3)

The 25-hydroxylcholecalciferol quantification was performed at Homerton Hospital laboratory, which is enrolled in the Charing Cross External Quality Control Schemes (DEQAS) for 25 hydroxycholecalciferol.

25 hydroxycholecalciferol was measured using the Waters ACQUITY UPLC XEVO Tandem quadrupole mass spectrometer (TQ MS) (Waters corp Milford MA), which uses the Ultraperformance liquid chromatography mass spectrophotometry/mass spectrophotometry (UPLCMS/MS) method. The manufacturers instructions were followed during the analysis. The Waters UPLC system consisted of a binary pump, binary solvent manager, auto sampler manager, column heater, Mass Lynx & Quanlynx software, vortex mixer Millipore 1L quick fit vacuum filter system, Varian Captiva 96 well 0.2 μm filter plate, Varian Captiva 96 well, 1mL collection plate, Varian vacuum manifold and pump for Captiva filter and photodiode array detector with a tandem quadrupole mass spectrometer equipped with an electrospray ionisation (ESI) source (MS/MS detector).

150 μL of calibrator, quality control samples and study specimens were added into a multiwell plate. 20 μL of internal standard containing 25(OH)D₃ and 25(OH)D₂ labelled with hexa-deuterated hydrogen (Synthetica) was added to each sample to correct for analytical loss of vitamin D molecules. The proteins in the samples were extracted by mixing with 150 μL 0.2M zinc sulphate (Sigma Aldrich) and 300 μL 80% methanol (Sigma Aldrich) and vortex mixing for 2 minutes. Vitamin D molecules were extracted by addition of 750 μL of hexane (Sigma Aldrich) followed by mixing and centrifugation for 5 minutes at 1500 relative centrifugal force (g). The layer of hexane was aspirated and dried by evaporation in a new microtitre plate.

The residue was re-dissolved in 200 μL of 80% methanol buffer and loaded into the Acquity UPLC where the analytes were separated in an ultra pressure liquid chromatography column. The eluate was loaded onto the LCMS/MS where it was ionised with ESI and ionised molecules produced were separated according to their mass charge ratio (m/z) in the first quadrupole. Entry into the collision cell resulted in collision-induced dissociation of the 25(OH)D₃ molecules facilitated by argon and collision energy to produce characteristic 25(OH)D₃ daughter ions that were detected in the third quadrupole. The third quadrupole allows ions with a specific mass to charge ratio to pass through to the detector.

The 25(OH)D3 daughter ions were expressed as a ratio of the internal standard 25(OH)D3 or 25(OH)D2 which was directly proportional to the concentration of the 25(OH)D3 in the sample.

2.1.7 Peripheral blood mononuclear cells (PBMC) preparation, positive selection of CD14+ cells and generation of monocyte-derived macrophages (MDM).

A volume of 150 ml whole blood was collected from normal healthy donors in heparinised (Biochrome) 50ml syringes after obtaining written informed consent. The blood was diluted 1:3 with phosphate buffered saline without calcium (Sigma Aldrich) and overlaid on a density gradient of lymphoprep (Axis-Shield) for centrifugation to extract peripheral blood mononuclear cells. Monocytes were extracted from PBMCs by positive selection using CD14 magnetic cell sorting (MACS) system (Miltenyi biotec) according to the manufacturer's instructions. This involved mixing the PBMC with magnetic microbeads coated with anti-CD14 (Miltenyi biotec) at a ratio of 1:5 (beads: cells) and incubation at 4°C for 15 minutes. The cells were washed twice to remove unbound beads and loaded into LS MACS column (Miltenyi biotec) that was placed in a MidiMACS separator magnetic field (Miltenyi biotec). CD14 positive cells (+) were retained in the column while the unlabelled cells passed through the column and were collected in tubes. The retained CD14+ monocytes were eluted into new tubes after rinsing of the column with MACS buffer.

The CD14+ monocytes were differentiated and matured into pro-inflammatory GM-CSF (M ϕ -1) and anti-inflammatory M-CSF macrophages (M ϕ -2) as described in (Verreck *et al.*, 2004). The monocytes were suspended in RPMI 1640 (Invitrogen) containing 10% heat inactivated autologous serum and 5 ng/ml of GM-CSF (Miltenyi biotech) for pro-inflammatory MDM or 20 ng/ml M-CSF (Miltenyi biotec) for anti-inflammatory MDM. The cells were plated into 6-well microtitre plates (Corning) at a concentration of 1×10^6 cells/ml and incubated at 37°C in 5% carbon dioxide for 6 days. On day 6 adherent MDM were gently removed using 0.25% Trypsin-EDTA (Biowest) and a cell scraper and were then counted after staining with trypan blue.

2.1.8 Flow cytometry analysis

Immunophenotyping of the M ϕ 1 and M ϕ 2 was performed using the 4 colour FACS Calibur (Becton Dickinson). M ϕ 1 and M ϕ 2 surface receptors were identified by use of flurochrome labelled monoclonal antibodies. 200 μ l of the cell suspension containing 2×10^5 MDM in phosphate buffered saline (PBS) containing 5% Foetal calf serum (FCS) (5% FCS/PBS) were plated per well into 96 well round bottom microtitre plates (Corning). The microtitre plate was centrifuged at 400g for 5 min and the supernatant was discarded. 50 μ l of 5% FCS/ PBS containing optimal volume of each appropriate antibody was added to the cell pellets and incubated for 15 minutes at 4°C in the dark (Table 1). Thereafter the cells were washed three times with 5% FCS/PBS to remove unbound antibody and then fixed for 20 minutes at 4°C with 100 μ l of cytofix buffer (BD Pharmingen). Excess cytofix buffer was washed from the cells with 200 μ l of 5% FCS/PBS and finally the cells were suspended in 200 μ l 5% FCS/PBS that was transferred to 5 ml FACS tubes (BD Pharmingen) for acquisition on the flow cytometer. Data analysis was performed using Flowjo version 9.5.3 (Tree star).

2.1.8.1 Determination of optimal antibody concentration

The optimal antibody concentration required for each antibody was titrated in order to determine the antibody volume needed for maximal staining of the monocyte derived macrophages with minimum background staining under the conditions of the experiment. Serial titrations of each antibody were used to stain the MDM as described in section 2.1.7.1. The samples were analysed by flow cytometry to determine the concentration at which the sample is near, but not above, the saturation point (Table 2.1).

Table 2.1 Antibody List

Instrument : BD FACS CALIBUR BD Pharmingen/557742/M5E2			
Antibody	Flouochrome	Optimal Volume	Vendor/Cat number/clone
CD14	PE-CY7	5ul	BD Pharmingen /557742/M5E2
CD1a	PE	5ul	BD Pharmingen /333167/SK9
CD282 (TLR2)	ALEXA 488	10ul	BD Pharmingen /558318/11G7
CCR5	PE CY7	5ul	BD Pharmingen /557752/207/CCR5
CD4	PE	5ul	BD Pharmingen /345769/SK3
CD15	FITC	5ul	BD Pharmingen /332778/MMA
CD16	PE	5ul	BD Pharmingen /555407/3G8
CD209	FITC	5ul	BD Pharmingen /551264/DCN46
CD11b	PE	5ul	BD Pharmingen /333142/D12
HLA-DR	FITC	5ul	BD Pharmingen /347400/L243
CD33	PE-Cy7	5ul	BD Pharmingen /333952/P67.6
IgG1 isotype control	ALEXA488	5ul	BD Pharmingen/ 557702
IgG2a isotype control	PE-CY7	5ul	BD Pharmingen/ 557907
IgG1 isotype control	PE	5ul	BD Pharmingen/ 555749
IgG2b isotype control	FITC	5ul	BD Pharmingen/ 555742

2.1.8.2 Calibration of the flow cytometer

The flow cytometer instrument settings, sensitivity and fluorescent compensation was optimised and monitored using the BD CALIBRITE beads and the FACSComp version 5.2.1 software before acquisition of samples according to the manufacturers instructions. Briefly it involved the analysis of FITC, PE, PERCP, APC and unlabelled beads. These adjusted the photomultiplier tube (PMT) voltages, compensation and sensitivity of all the detectors according to the reference target values (Table 2.2).

Table 2.2 Flow cytometer set up

Instrument	BD FACS CALIBUR					
Laser lines	488nm Blue laser					635nm Red laser
Emission filters	530/30	530/30	575/26	780/60	PerCP	670/14
Flouorochrome	FITC	Alexa fluor 488	PE	PE-CY7	682/33	APC

2.1.8.3 MDM controls

M ϕ -1 and M ϕ -2 single stained with TLR-2 Alexa flour 488, CD11b PE, CD33-CY7, and CD4-APC and were used for compensation and analysis of the MDM. The unstained MDM were used to optimise the resolution of the MDM and evaluate autofluorescence and confirm the region or channel in which the unstained monocyte derived macrophages were placed. Isotype matched controls were used for the evaluation of non-specific staining and to set the negative and positive analysis regions of the cell populations using quadrant markers.

2.1.8.4 Acquisition of data

The BD cell quest [™] Pro software version 5.2.1 was used for creation of the acquisition template with the scatter plots forward scatter (FSC) vs. side scatter (SSC), FL1 vs. FL2, FL2 vs. FL3 and FL3 vs. FL4. The MDM were located using FSC and SSC and gated accordingly. The gating strategies used to analyse the subsets of the MDM are shown in the results section of chapter 5.2.2.1.

2.1.9 Preparation of *M. tuberculosis* stock

The following frozen glycerol stocks of *M. tuberculosis* strains were used: H37Rv, LAM3 and HN878 and the stock of each strain was prepared by adding 100 μ l of each *M. tuberculosis* strain into 10 ml of Middlebrook 7H9 broth (BD Pharmingen) supplemented with 0.2% glycerol, 10% albumin dextrose and catalase (ADC) (BD Pharmingen) and 0.05% tween 80 (Sigma Aldrich) and incubated for 10 days at 37°C.

Thereafter 1ml of the culture was subculture in 100 ml Middlebrook 7H9 broth supplemented with 0.2% glycerol and 10% ADC. After 10 days, single cell stock of each specific *M. tuberculosis* strain was prepared through first sedimenting the *M. tuberculosis* cell suspension by centrifugation for 5 minutes at 2500 revolutions per minute (rpm) and discarding the supernatant. The bacterial cells were separated into single cells by shaking vigorously with 3 mm glass beads (Sigma-Aldrich) followed by mixing with 6 ml of PBS. After leaving the tube to stand for 5-10 minutes, the upper 5 ml of suspension was harvested and spun for 10 minutes at 1400g. This was followed by harvesting of the upper 4.5ml into a tube containing 500 µl of 50% glycerol and mixed. The single cell stocks was then aliquoted into 2 ml cryol tubes and stored at -80°C. To determine the titer of each strain stock, ten fold serial dilutions of each strain were prepared using PBS containing 0.01% tween 80 and colony forming units (CFU) were determined by plating the serial dilutions in multiple replicates on Middlebrook 7H11 agar supplemented with 10% oleic acid, albumin, dextrose and catalase (OADC) and 0.5% glycerol. The culture plates were incubated at 37°C for 3-4 weeks.

2.1.10 Growth curves

The growth curves of the *M. tuberculosis* strains H37Rv, LAM3 and HN878 were determined by inoculation of 100 µl of each *M. tuberculosis* strain stock in 10 mls of Middlebrook 7H9 broth supplemented with 10% ADC and 0.05% tween 80. This was incubated at 37°C and optical density was measured using a Novaspec II spectrophotometer (Biochrom Ltd) at 600 nm everyday until a OD₆₀₀ of between 0.6 to 0.8 was reached when the bacteria are in the log phase of growth. The *M. tuberculosis* culture was then sub-cultured into 100 ml of Middlebrook 7H9 supplemented with 0.2% glycerol, 10% ADC and 0.05% Tween 80 at an OD₆₀₀ of 0.01. The OD₆₀₀ was monitored daily up to an OD₆₀₀ of 0.4 to 0.5 and an aliquot was taken and inoculated on blood agar plate to exclude for contamination.

The OD₆₀₀ 0.4 culture was then sub-cultured at an OD₆₀₀ of 0.01 in triplicates of 100 ml of Middlebrook 7H9 broth supplemented with 0.2% glycerol, 10% ADC and 0.05% Tween 80. The OD₆₀₀ was measured daily at the same time for up to 3 weeks and the growth curve was drawn using the absorbance and number of days in culture.

2.1.11 Preparation of the HIV-1 BAL

The CCR5 tropic HIV-1 BAL was prepared by propagation in CD14 negative peripheral blood lymphocytes left over after separation of CD14+ monocytes. The peripheral blood lymphocytes were activated by culturing them for 3 days in RPMI 1640 containing 20% Foetal calf serum (FCS) (Sigma Aldrich) and 0.5 µg/ul phytohemagglutinin (PHA) (Sigma-Aldrich). These were infected with HIV-1 BAL at multiplicity of infection (MOI) of 1:1 and cultured in RPMI 1640 with 20% FCS and 20U/mL of IL-2 (PeproTech). Cell culture supernatants were harvested every third day, leaving 1 ml in culture, and were filtered through a 0.45 µm Millipore filter (Corning). Additional PHA activated peripheral blood lymphocytes were added to keep the cell density at 1×10^6 cell/ml. The harvested cell culture supernatants were used to infect 6 day MDM cultures MOI of 1:1 and the media refreshed after 16 hrs. Every week supernatants from the infected MDM were harvested and centrifuged at 400g for 5 minutes and filtered through a 0.45 µm Millipore filter to remove cellular debris and stored at -80 °C. The virus suspensions were ultracentrifuged through 20% sucrose buffer and resuspended in RPMI 1640 with 5% AB normal human serum and stored at -80°C for subsequent infection of MDM.

The titre of the virus suspension was determined through titration on neural pentraxin 2 (NP2) cells transfected with CD4 and CCR5 or CXCR4. The NP2 cells were cultured in Dulbecco's modified eagle medium (GIBCO Invitrogen) with 5% FCS, 1 µg/ml puromycin (Sigma-Aldrich) and 2.5 mg/ml G418 (Sigma Aldrich). The cells were infected with serial log fold dilutions of viral stocks and incubated for 2 hrs at 37°C in 5% CO₂ before removing the inoculum and replacing the media. The immunostaining for p24 was used for detection of infection after 72 hrs as described below.

2.1.11.1 Intracellular p24 staining

Media was removed and the cell layer was covered with ice-cold methanol: acetone at ratio of 1:1 vol:vol for 5 minutes and washed 3 times with PBS. The NP2 cells were incubated with mouse anti-HIV gag (p24) monoclonal antibodies (E365/366) (National Institute of Biological Standards and Control UK) for 2 hrs at room temperature. This was followed by incubation with goat anti-mouse immunoglobulin conjugated to galactosidase (gal) (Southern Biotechnology Associates) for 1 hr at room temperature. The gal substrate solution was then added and incubated overnight at 37°C to develop a blue coloured solution. The virus titre was calculated by counting the number and proportion of positively stained cells under the microscope (forming units/ml).

2.1.12 Co-infection of MDM with HIV and *M. tuberculosis* strains

For the investigation of the effect of HIV/TB co-infection on growth *M. tuberculosis* strains and HIV replication, MDM were generated from blood from 5 normal healthy donors at the Institute of Infectious Diseases and Molecular Medicine, University of Cape Town. The MDM were plated into flat bottom 96 well microtitre plates (Corning) at a concentration of 8×10^5 cells per well. They were then infected with HIV-1 BAL at a multiplicity of infection MOI 1:3 in triplicates and the plates were incubated at 37°C in 5% carbon dioxide for 7 days with refreshment of the media every 3 days. On day 7, the MDM were infected with *M. tuberculosis* strains H37Rv, LAM3 and HN878 at MOI 1 in triplicates. The culture plates were incubated at 37°C in 5% carbon dioxide for 7 days and supernatants were collected after 4 hrs, 24 hrs, 96 hrs and 168 hrs for p24 measurement and stored at -80°C. After 4 hrs, 24 hrs, 96 hrs and 168 hrs, extracellular bacteria were removed from adherent cells by washing and the cells were then lysed with deionised water containing 0.05% tween 80 (Sigma-Aldrich). The suspension was serially diluted, and the highest 3 dilutions were plated in triplicate on Middlebrook 7H11 agar (BD Pharmingen) supplemented with 0.5% glycerol (Sigma-Aldrich) and 10% OADC (BD Pharmingen). The plates were incubated at 37°C for 3-4 weeks and *M. tuberculosis* CFU were enumerated.

2.1.13 HIV/TB co-infection in presence of vitamin D molecules

For the determination of the effect of vitamin D molecules on HIV replication in the presence and absence of *M. tuberculosis* 12 healthy donors were used and 6 healthy donors were used for determination of effect of vitamin D molecules on *M. tuberculosis* growth.

For the investigation of effect of vitamin D molecules on HIV replication and *M. tuberculosis* growth, CD14⁺ monocytes were differentiated into M ϕ 1 in RPMI 1640 containing 5ng/ml GM-CSF and either 100nm 1,25 dihydroxycholecalciferol (1,25(OH)₂D3) (Sigma-Aldrich) or 100nm 25-hydroxycholecalciferol (Sigma- Aldrich) and 0.01% ethanol (Sigma-Aldrich) as vehicle. M ϕ 2 were differentiated in RPMI 1640 containing 20 ng/ml M-CSF and either 100 nm 1,25 dihydroxycholecalciferol (1,25(OH)₂D3) or 100 nm 25-hydroxycholecalciferol and 0.01% ethanol as vehicle. The monocytes under each condition were plated in triplicates at a concentration of 1×10^6 cells/ml in a 6 well microplate and incubated at 37°C in 5% carbon dioxide for 6 days.

On day 6 adherent MDM were removed gently using 0.25% Trypsin-EDTA-and a cell scraper. MDM were counted and plated into flat bottom 96 well microtitre plates (Corning) at a concentration of 8×10^5 cells in RPMI 1640 containing 5% AB serum (Biowest) and supplemented with the appropriate corresponding vitamin D molecules followed by infection with HIV-1 BAL at MOI 1:3 in triplicate. The plates were incubated at 37°C in 5% carbon dioxide for 7 days with media refreshment by removal of the supernatants and addition of the new RPMI media containing vitamin D molecules every 3 days. The 72 hrs and day 6 supernatants were stored at -80 °C. On day 7 the MDM were infected with *M. tuberculosis* strain, H37Rv at a MOI of 1:1. The culture plates were incubated at 37°C in 5% carbon dioxide for 7 days. Supernatants were collected after 72 hrs and day 6 and were stored at -80°C for p24 measurement. Extracellular bacteria were removed from adherent cells by washing and the cells were then lysed with deionised water.

The suspension was diluted and plated on Middlebrook 7H11 agar supplemented with 0.5% glycerol and 10% OADC after 4 hrs, 24 hrs, 96 hrs and 168 hrs for determination of *M. tuberculosis* CFU. The plates were incubated at 37°C for 3-4 weeks after which CFUs were enumerated.

2.1.14 Measurement of p24 in supernatants

Supernatants were incubated with 100 µl of 10% Triton X (Sigma-Aldrich) at 37°C for 2 hrs to lyse the HIV. Supernatants were then filtered twice through Millipore 0.22 µm durapore 96 well filter plates (Corning) to remove any *M. tuberculosis*. The p24 levels in the supernatants were measured using the HIV-1 p24^{CA} antigen capture assay kit (SAIC Frederick) following the manufacturers instructions. Briefly, 100 µl of samples and 1:2 serial dilutions of standards, were added to 96 well plates coated with monoclonal antibody to HIV-1 p24, and blocked with 0.5% casein solution. After 2 hrs incubation at 37°C, the plates were washed and incubated with 100 µl of 1:200 dilution of primary rabbit anti-HIV-1 p24 antibody at 37°C for 1 hr. The plates were washed and incubated with 100 µl of 1:100 dilution of secondary goat anti-rabbit IgG conjugated with horseradish peroxidase (SAIC Frederick) for 1 hr at 37°C. After the plates were washed TMB substrate (KPL) solution was added and caused an enzymatic reaction with production of a coloured soluble product. The reaction was stopped by addition of 1N HCL (Sigma-Aldrich) and the absorbance of coloured product was measured with plate reader at a wavelength of 450 nm with a reference filter of 650nm. The colour that developed was directly proportional to the quantity of p24. Concentration of p24 in supernatants was calculated from the standard curve.

2.1.15 Statistical analysis

Data for the vitamin D and tuberculosis cross-sectional study was analysed using the SPSS version 20 (SPSS Inc.) and GraphPad Prism version 5 (GraphPad) software packages. Contingency tables were analysed using the Chi-squared test and Fisher's exact test when the expected frequencies were less than 10.

The Mann-Whitney test was used to compare median values while the unpaired t-test was used to compare means. The Bonferroni test was used for multiple comparisons. Spearman's R was used for the calculation of correlation between age, BMI, CD4 and serum 25(OH)D3. Multivariate analysis of correlates of serum 25(OH)D3 contrasts were conducted using multiple linear regression and p-values less than 0.05 were considered to be significant.

For the *in vitro* HIV/TB macrophage co-infection experiments GraphPad Prism version 5 was used for statistical analysis of the results. One-way ANOVA was used for the analysis of independent variables with more than two conditions. The effects of two or more independent variables with multiple conditions were analysed by two-way repeated measure ANOVA. The Bonferroni test and Dunn's test with correction for multiple comparisons were used for post hoc pairwise comparisons to identify the specific significant differences between each pair.

2.2 Materials

Table 2.3 Materials and reagents used in methods described above

Reagents and materials	Supplier and product number
BD Falcon™ Polystyrene round bottomed tubes, 5 ml, 12x75 mm style	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA#352054
FACS flow	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA
FACS clean	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA
FACS rinse	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA
BD cytofix/Cytoperm™	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA #51-2090KZ
Small 25 cm cell scraper	Corning, NY 14831, USA CLS3010
RPMI 1640	Sigma-Aldrich, South Africa #R8758
Trypsin EDTA (0.25% 1X)	BioWest, France L0931-100
Human serum AB male	Biowest, France 54190-100
Round bottom 96 well microtitre plates	Corning, NY 14831, USA #14831
PBS with Ca2+	Life Technologies, South Africa, # 14025-050

Reagents and materials	Supplier and product number
PBS without Ca ²⁺	Sigma-Aldrich, South Africa # D8537
Middlebrook 7H9 broth	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA
Middlebrook ADC enrichment	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA 211886
Middlebrook OADC enrichment	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA 211887
H37Rv strain	ATCC strain from IIDMM university of Cape Town
HN878 strain	Donation from the National Institute of Medical Research (UK)
LAM3 strain	Clinical strain from patient from Khayelitsha Clinic
Tween 80	Sigma-Aldrich, South Africa # P1754
Millex syringe filter 0.22 µm Durapore PVDF	Sigma-Aldrich, South Africa # F7648-50
Glycerol	Sigma-Aldrich, South Africa # G5516-1L
Millex syringe filter 0.45 µm Durapore PVDF	Sigma-Aldrich, South Africa # Z355518-50
Solid-glass beads borosilicate, diameter 3 mm	Sigma-Aldrich, South Africa # Z143928
1L polystyrene bottles	Corning, NY 14831, USA # 430518
250ml polystyrene bottles	Corning, NY 14831, USA # 430281
Biolite 175 cm ² Flask	Thermo Fisher Scientific, USA # 130190
Biolite 175 cm ² Flask	Thermo Fisher Scientific, USA # 130190
50ml Falcon tubes	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA #352070
Tuberculin Purified Protein Derivative (PPD)	Statens Serum Institute, DK-2300, Copenhagen S, Denmark #2391
BD CaliBRITETM 3 three-color-kit	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA #340486
Reagents and materials	Supplier and product number
BD CaliBRITETM APC Beads	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA #340487
Trypan blue 0.4%	Sigma-Aldrich, South Africa #T8154
Middlebrook 7H11 agar	Becton Dickson Biosciences, Pharmingen, San Diego, CA, USA

Reagents and materials	Supplier and product number
L-shaped spreaders	Sigma-Aldrich, South Africa # Z376779
Triton X 100	Sigma-Aldrich, South Africa # X100
96 well filter plates 0.2 µm PVDF hydrophilic membrane	Sigma-Aldrich, South Africa # CLS3505
Fetal calf serum, endotoxin free	Delta Bioproducts, Highveld Biological, Lyndhurst, South Africa #14-501-BI
Fugene 6 transfection reagent	Roche Applied Science # 11 815 091 001
Optimem media (without additives)	Life Technologies, UK # 11058-021
Sucrose	Sigma-Aldrich, South Africa # S0389
70% Ethanol	Sigma-Aldrich, South Africa # E7023
Lymphoprep TM	Axis-Shield, Oslo Norway # 1114547
Heparin (5000 U/ml)	Biochrom AG Berlin # L6510
Bovine serum Albumin	Sigma-Aldrich, South Africa # A7906
Sodium Pyruvate 100 mM	Life Technologies, South Africa #11360-039
Disodium EDTA 18.61g	Sigma-Aldrich, South Africa # E5134
NaOH	Sigma-Aldrich, South Africa # S5881
MidiMACS separator	Miltenyi Biotec # 130-042-302
LS MACS separation columns	Miltenyi Biotec # 130-042-302
Human GM-CSF premium grade (100 µg)	Miltenyi Biotec # 130-093-866
Human M-CSF premium grade (100 µg)	Miltenyi Biotec # 130-096-492
HIV-1 p24 Antigen Capture Assay Kit	SAIC Frederick, USA
20X Wash concentrate Buffer	KPL, Washington DC, USA 50-63-01
Tween 20	Sigma-Aldrich, South Africa # P1379
Normal mouse serum (NMS)	Sigma-Aldrich, South Africa # M5905
Normal goat serum (NGS), GIBCO	Life Technologies, South Africa, #16210064
SureBlue TMB Microwell Peroxidase substrate solution.	KPL, Washington DC, USA # 52-00-02
Hydrochloric Acid	Sigma-Aldrich, South Africa # H1758
Analytical UPLC column- Waters Acquity UPLC – BEH C18, 1.7 µm, 2.1 x 50 mm.	Waters, USA - # 186002350
Methanol Hypersolve grade.	VWR, Radnor, USA #152507P
Ammonium Acetate	Sigma-Aldrich, South Africa # 09689
Formic Acid	Sigma-Aldrich, South Africa # 33015
Deuterated 25 Hydroxyvitamin D3 (5 mg)	Synthetica, Oslo, Norway
25 Hydroxyvitamin D2 & D3 calibrator	Chromsystems, Munich Germany # 38033
25 Hydroxyvitamin Vitamin D3 bi-level control (I+II)	Chromsystems, Munich Germany # 0028

Chapter 3: Reciprocal Seasonal Variation In Vitamin D Status And Tuberculosis Notifications In Cape Town, South Africa.

3.1 Literature review

3.1.1 Vitamin D status

Vitamin D nutritional status is determined by measurement of circulating 25(OH)D₃ and 25(OH)D₂ levels in plasma or serum. These metabolites are suitable, because of their stability and longer half-life, the plasma half-life of 25(OH)D₃ is 15 days while that of 1,25(OH)₂D₃ is of 10-20 hours (Jones, 2008). The active metabolite 1,25(OH)₂D₃ is not a good measure of vitamin D status as it is tightly regulated throughout a wide range of 25(OH)D₃ concentration. There is some debate about 25(OH)D₃ levels which signify vitamin D deficiency and sufficiency since recommendations for higher cut-off values are based on observational epidemiological studies, which might be confounded rather than on clinical trials. The general consensus among experts is that vitamin D deficiency is defined as 25(OH)D₃ levels less than 50 nmol/L, vitamin D insufficiency levels from 51 nmol/L to 74nmol/L and vitamin D sufficiency levels above 75 nmol/L. Vitamin D intoxication occurs when the 25(OH)D₃ levels are above 375 nmol/L (Holick, 2007).

Competitive immunoassays and chromatographic direct detection methods are the two main methods used for 25(OH)D₃ and 25(OH)D₂ quantification. The chromatographic methods such as liquid chromatography tandem mass spectrophotometry (LC-MS/MS) are more accurate and precise. They begin with the deproteinisation, lipid extraction and chromatographic separation of the 25(OH)D₃ and 25(OH)D₂ in serum samples. Quantification in LC-MS/MS involves atmospheric pressure ionisation of 25(OH)D₃ molecules and measurement by mass detection. Their major disadvantage is that they require expensive equipment and technical expertise (Lensmeyer *et al.*, 2006, Wallace *et al.*, 2010).

3.1.2 Vitamin D deficiency

Globally it has been noted that there is an increasing prevalence of vitamin D deficiency, which is due to a variety of causes, mainly related to lifestyle changes in most populations. The vitamin D status of populations is dependent on availability of sunlight, nutritional provision and the underlying disease states. Sunlight is generally the main source of vitamin D rather than diet. As the latitude increases to above 30°N or 30°S the need for dietary vitamin D supplementation increases especially in winter (Holick, 2007). The high prevalence of vitamin D deficiency has been found to be associated with increased economic burden of disease in Europe (Grant *et al.*, 2009). The common assumption that vitamin D deficiency is not prevalent in Africa due to the high availability of sunlight might have contributed to very few studies on vitamin D being performed in Africa. The available data show that the vitamin D status of African populations are variable depending on region. According to the current acceptable limits of vitamin D sufficiency, there is wide spread vitamin D deficiency and insufficiency in different population groups in Africa, which range from healthy adults to pregnant women (Prentice *et al.*, 2009).

Factors that influence vitamin D status in Africa are low calcium intake, malnutrition, fluorosis, tropical enteropathy, tuberculosis, Human Immunodeficiency Virus infection (HIV), malaria, non-communicable diseases and vitamin D inadequate diet (Prentice *et al.*, 2009). More recently vitamin D deficiency has been shown to result in increased risk and susceptibility to infectious diseases and non-communicable diseases (Heaney, 2008).

3.1.3 Vitamin D and Tuberculosis

From the early 1700s, vitamin D was used for the treatment of tuberculosis in Europe. Various sanatoria were set up in various parts of the world, some in the mountains where patients were exposed to fresh air and sunlight for vitamin D treatment. Later on Cod liver oil, which contains high vitamin D levels, was used for treatment of tuberculosis. Vitamin D and UV light lamps have been successfully used in the treatment of cutaneous tuberculosis (Moller *et al.*, 2005).

The discovery of antibiotic drugs for treatment of tuberculosis resulted in the use of vitamin D treatment being less preferable.

In vitro experiments by Rook *et al* in the early 80s demonstrated that infection of macrophages with *M. tuberculosis* in the presence of 1,25(OH)₂D₃ resulted in growth restriction of *M. tuberculosis* (Rook *et al.*, 1986). The same results were corroborated by Crowle and colleagues, who also showed that supplementation of macrophages with 1,25(OH)₂D₃ increased their ability to inhibit growth of *M. tuberculosis* (Crowle *et al.*, 1987). In 2006 Liu and colleagues were able to demonstrate that the ability of 1,25(OH)₂D₃ to kill *Mycobacterium tuberculosis* was mediated through its stimulation of secretion of the antimicrobial peptide cathelicidin (Liu *et al.*, 2006).

More *in vitro* studies using monocytes and macrophages demonstrated that binding of *M. tuberculosis* antigens on macrophage TLR2/1 results in increased expression of VDR and CYP27B1 which causes increased synthesis of 1,25(OH)₂D₃ and induces the antimicrobial activities against *M. tuberculosis* (Fabri *et al.*, 2011). Apart from the direct effects of antimicrobial peptides, 1,25(OH)₂D₃ mediates inhibition of *M. tuberculosis* growth by autophagy and production of reactive oxygen species and reactive nitrogen intermediates (Yuk *et al.*, 2009, Sly *et al.*, 2001, Thoma-Uszynski *et al.*, 2001). Furthermore, 1,25(OH)₂D₃ has been shown to modulate the immune response by inhibiting expression and secretion of pro-inflammatory cytokines such TNF- α , IL-12 and IL-17 (Ikeda *et al.*, 2010, Daniel *et al.*, 2008, Sigmundsdottir *et al.*, 2007, Jeffery *et al.*, 2009) and matrix metalloproteinases (MMP) 7, 9 and 10 during *M. tuberculosis* infection (Coussens *et al.*, 2009). This might be important in the reduction of tissue damage during tuberculosis.

A number of studies in different geographic areas have shown association between vitamin D deficiency and tuberculosis. Early studies in London showed an association between vitamin D deficiency and tuberculosis among Gujarati Indians (Wilkinson *et al.*, 2000). In the African setting, studies in Guinea-Bissau, Malawi and Uganda have shown increased prevalence of vitamin D deficiency in tuberculosis patients (Nansera *et al.*, 2011, Banda *et al.*, 2011, Wejse *et al.*, 2007). In contrast two studies

in Indonesia and South Korea, found no significant difference between vitamin D levels of tuberculosis patients and controls (Grange *et al.*, 1985, Koo *et al.*, 2012). Nevertheless, a recent meta-analysis by Nnoaham and Clarke, concluded that low vitamin D levels were associated with a higher risk of developing active tuberculosis (Nnoaham and Clarke, 2008). Moreover patients with vitamin D deficiency have been shown to be at increased risk of autoimmunity in *M. tuberculosis* infected individuals (Li *et al.*, 2012).

Apart from the association between 25(OH)D3 deficiency and active tuberculosis, sufficient 25(OH)D3 levels have been shown to protect against progression and development of active tuberculosis. Serum 25(OH)D3 concentration has been associated with increased risk for latent TB infection among African immigrants. The study showed that patients with latent TB who were vitamin D deficient were at high risk of developing active tuberculosis (Gibney *et al.*, 2008). A 4-year longitudinal cohort study in Pakistan demonstrated that vitamin D deficiency in household contacts of patients with tuberculosis was associated with 5 fold-increased risk of progression to active tuberculosis. The limitations of this study were small sample size, and there was a lack of information on BMI, diet and exposure to sunlight (Talat *et al.*, 2010).

In addition sufficient 25(OH)D3 (≥ 75 nmol/L) was associated with protection against tuberculin skin test (TST) conversion (Arnedo-Pena *et al.*, 2011). There was also a reduced risk of TST conversion in children whose serum 25(OH)D3 levels increased to more than 20 ng/ml after supplementation (Ganmaa *et al.*, 2012). Furthermore Martineau and colleagues were able to demonstrate that whole blood from TB contacts that had received vitamin D supplementation was able to restrict growth of *Bacille Calmette-Guerin* (BCG) (Martineau *et al.*, 2007c).

VDR polymorphism has been associated with susceptibility to active tuberculosis and response to treatment.

The Taq1 and Fok1 restriction fragment length polymorphisms (RFLP) have been associated with susceptibility to active tuberculosis and early microbiologic clearance of pulmonary tuberculosis during treatment (Wilkinson *et al.*, 2000, Roth *et al.*, 2004, Martineau *et al.*, 2011). The taq1 tt genotype was associated with resistance to *M. tuberculosis* infection among West Africans (Bellamy *et al.*, 1999). A systematic review and meta-analysis by Gao *et al.*, points to a possible role of VDR polymorphism as a risk factor in the development of tuberculosis (Gao *et al.*, 2010). The vitamin D binding protein (DBP) polymorphisms play a role in bioavailability of vitamin D. The DBP variants Gc2-1S and Gc2-2 have low affinity to vitamin D compared to Gc1F-1F (Chun *et al.*, 2010). The Gc2-2 genotype is associated with increased susceptibility to active TB among Gujarati Indians due to its inability to transport sufficient amounts of vitamin D to target tissues (Martineau *et al.*, 2009a). In addition CYP27B1 polymorphism might have a role in immunity to tuberculosis, as it has been associated with Vitamin D deficiency in other infectious diseases such as Hepatitis C (Lange *et al.*, 2011).

Vitamin D supplementation studies have not always produced the expected results due to a number of reasons related to their study designs. Supplementation of 0.25mg vitamin D/day for 6 weeks in TB patients receiving conventional therapy resulted in higher rates of sputum smear conversion in the treatment group compared to the placebo group ($p=0.002$) (Nursyam *et al.*, 2006). Morcos *et al.*, also reported faster resolution of TB symptoms in children treated with 1,000 IU of vitamin D daily as an adjunct to standard TB therapy (Morcos *et al.*, 1998). Both studies did not report baseline and follow up vitamin D levels in order to determine the adequacy of the supplementation. In contrast, a randomized double-blind placebo trial by Wejse *et al.*, (2009) in Guinea-Bissau, showed no effect of supplementation on clinical outcomes or mortality amongst TB patients, the 2 doses of 100000 IU vitamin D3 which were administered was low since there was no significant increase in serum 25(OH)D3 levels suggestive of insufficient supplementation or mixed up randomisation codes.

In a recent multicentre, randomized, double-blind, controlled trial *Martineau et al*, (2011) reported that only patients with the tt genotype of the taq1 VDR polymorphism had faster sputum culture conversion after receiving standard tuberculosis treatment and vitamin D. There was no significant difference in rate of sputum culture conversion between the treated group and the placebo group (Martineau *et al.*, 2011). Nevertheless, vitamin D3 adjunctive therapy enhanced the clearance of the pro-inflammatory responses in tuberculosis patients by inhibiting the secretion of pro-inflammatory cytokines and chemokines, and reduced the immunosuppressive effect of tuberculosis antimicrobial therapy and which could be important in the prevention of severe tissue damage. On the other hand, it also enhanced the clearance of monocytosis, lymphopenia, hyperchemokinaemia and hyperkalaemia (Coussens *et al.*, 2012). Furthermore vitamin D supplementation has been associated with low risk of all cause mortality in a meta-analysis of vitamin D randomized controlled trials (Autier and Gandini, 2007).

3.1.4 Vitamin D and HIV

A high prevalence of vitamin D deficiency has been reported in HIV infected patients. Comparison of levels of vitamin D metabolites in HIV-infected individuals and appropriate control patients has shown that HIV infected patients have lower mean levels of 1,25(OH)₂D₃ and 25(OH)D₃ which has been related to the increased occurrence of hypocalcaemia attributed to increased bone turnover during HIV progression (Teichmann *et al.*, 2003, Kuehn *et al.*, 1999, Teichmann *et al.*, 2000). The severity of vitamin D deficiency increases as HIV infection advances to acquired immunodeficiency syndrome (AIDS) (Haug *et al.*, 1998a).

Increased prevalence of vitamin D deficiency in HIV infected individuals has been associated with use of antiretroviral drugs and effects of HIV on bone health (Childs *et al.*, 2012). HIV positive individuals have higher propensity to developing osteoporosis and osteopenia, conditions commonly associated with vitamin D deficiency (McComsey *et al.*, 2007, Mondy *et al.*, 2005).

This is because HIV proteins increase osteoclastic activity (Fakruddin and Laurence, 2005) and decrease bone formation by promoting osteoblastic apoptosis (Gibellini *et al.*, 2008).

Factors that lead to vitamin D deficiency during HIV infection include increases in chronic inflammation, immune activation, secretion of pro-inflammatory cytokines like TNF- α and IL-6 (Haug *et al.*, 1998a) and increased utilisation of vitamin D by macrophages and lymphocytes as disease progresses (Villamor, 2006). The over activation of TNF- α in HIV infected persons inhibits the stimulatory effect of PTH on 1 α -hydroxylase (Villamor, 2006, Haug *et al.*, 1994). In addition malabsorption of dietary vitamin D, reduced exposure to sunlight, and use of ARVs NNRTIs and PIs also contribute to vitamin D deficiency during the course of HIV infection (Cozzolino *et al.*, 2003, Bout-Van Den Beukel *et al.*, 2008). Vitamin D deficiency in HIV infected individuals might result in their increased susceptibility to active tuberculosis (Figure 3.1).

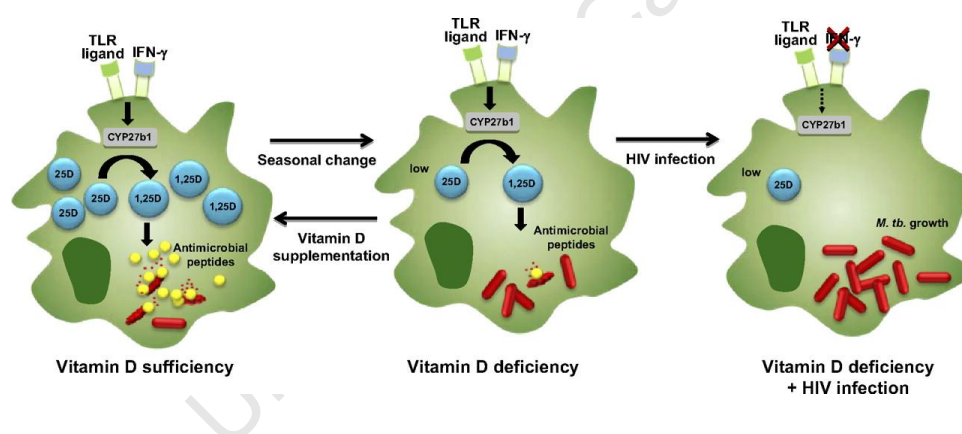


Figure 3.1 Vitamin D and the macrophage antimicrobial response to TB (Realegeno and Modlin, 2011).

Sufficient 25(OH)D3 levels(>32 ng/ml) have been shown to result in better outcomes in HIV infected women (Mehta *et al.*, 2010). Studies on the role of vitamin D on HIV replication are inconclusive. Some studies have shown that vitamin D increases HIV replication while others have shown that it inhibits HIV replication (Pauza *et al.*, 1993, Nevado *et al.*, 2007).

Cathelicidin (LL-37) an antimicrobial peptide secreted in response to vitamin D stimulation of cells has been shown to have immunomodulatory effects on HIV. It has been demonstrated to inhibit HIV replication in primary PBMC and CD4 T cells, through its effects on the target cells not the HIV (Bergmana *et al.*, 2007). Defensins have inhibitory effects on HIV replication, for example β -defensins inhibit HIV-1 infection by directly binding to HIV-1 virus and by down regulation of HIV fusion receptor CXCR4 (Quinones-Mateu *et al.*, 2003) and inhibition of intracellular replication (Sun *et al.*, 2005).

There are few studies on vitamin D supplementation in HIV infected individuals. Most of the studies focus on its importance on bone health in the HIV infected. Bimonthly administration of 100 000 IU of cholecalciferol and 1g calcium daily resulted in increased 25(OH)D3 levels >30ng/L in most of the patients and did not affect CD4 or viral load in the 12 months of the study. In addition supplementation did not have any adverse effects on the HIV infected participants (Arpadi *et al.*, 2009).

Vitamin D deficiency has been shown to be a major risk factor in progression of latent tuberculosis to active tuberculosis. In the case of HIV, vitamin D is proving to be more important as more studies are pointing towards its role towards prevention of disease progression to AIDS. Few studies have been done to investigate the role of vitamin D deficiency during HIV/TB co-infection in high disease burden settings like Cape Town South Africa. Although Cape Town is sunny and close to the sea with a lot of fish, there might be a high prevalence of vitamin D deficiency because of high disease burden due to HIV and tuberculosis (TB) and changes in life style as well as the fact that antiretroviral drugs (ARVs) and tuberculosis drugs interfere with vitamin D metabolism (Brodie *et al.*, 1982, Childs *et al.*, 2012).

Seasonal variation in rates of tuberculosis incidence has been noted in Cape Town. There are reports of increases in new cases, worsening of conditions of TB patients and reactivation of latent TB during late winter and early spring in the Western Cape. Previous studies have noted increased cases of tuberculosis in early spring after winter in children (Schaaf *et al.*, 1996). Pettifor *et al.*, 1996 demonstrated a decrease in Vitamin D synthesis using sunlight between April and September.

The Vitamin D synthesis from sunlight in Cape Town was reported to be 3 times less than that in Johannesburg during the same period (Pettifor *et al.*, 1996). The high prevalence of tuberculosis transmission and disease might be due to deficient innate immunity for surveillance of *M. tuberculosis* due to vitamin D deficiency. Therefore we sought to determine the prevalence of vitamin D deficiency in HIV infected and uninfected patients with tuberculosis in Cape Town, South Africa. This is the first study to investigate the association of vitamin D deficiency with tuberculosis in this population and settings.

3.2 Results

3.2.1 Characteristics of study participants

A total of 480 participants were recruited; 251 were HIV uninfected and 229 were HIV infected. The patients were further stratified into three clinical groups; 192 active tuberculosis (ATB), 178 latent tuberculosis infection (LTBI) and 110 diseases other than tuberculosis groups (OI). The demographic and clinical characteristics of the HIV uninfected study participants stratified by tuberculosis status are shown in Table 3.1.

In the HIV uninfected the comparison of ATB vs. OI revealed that diagnosis of active tuberculosis was associated with younger age ($p=0.01$), lower BMI ($p=0.02$), male sex ($p=0.001$), black race ($p<0.001$) and sampling between October to March ($p=0.02$). The latent TB vs. active TB comparison indicated that a diagnosis of active tuberculosis was associated with older age ($p<0.001$), low BMI ($p<0.001$), fewer years of education ($p<0.001$) and being a male ($p<0.001$). The ethnic group of 3 latent TB patients was missing and the season of collection unknown for one patient (Table 3.1)

Table 3.1 Demographic and clinical characteristics of HIV un-infected study participants (n=251).

HIV uninfected n=251					
Variables	OI (n=55)	LTBI (n=103)	ATB (n=93)	p-values	
				ATB vs. OI	LTBI vs. ATB
Median age, years (IQR)	38.9 (28.9 to 52.2)	22.65 (19.65 to 26.0)	31.7 (25.8 to 42.3)	0.010	<0.001
Median BMI, kg/m² (IQR)	22.5 (18.7 to 25.1)	23.0 (21.4 to 29.0)	20.1 (18.1 to 22.5)	0.02	<0.001
Education, Grade (IQR)	9 (7 to 11)	11 (10 to 12)	10 (7 to 11)	0.41	<0.001
Sex, n (%)					
Male	22(40)	42(40.8)	64(68.8)	0.001	<0.001
Females	33(60)	61(59.2)	29(31.2)		
Ethnic group					
Black	23	103	89	<0.001	0.05
'Coloured'	32	0	4		
Sampling					
Apr - Sept	34	39	39	0.02	0.52
Oct - Mar	21	64	53		

Table 3.2 shows the demographic and clinical characteristics of the HIV infected participants stratified according to tuberculosis status. The demographics and clinical characteristics were equally distributed for the OI and active TB groups. In the comparison of the latent TB vs. active TB diagnosis; active TB was associated with low BMI ($P<0.001$), low CD4 count ($p<0.001$) and male sex ($p=0.04$).

Table 3.2 Demographic and clinical characteristics of HIV infected study participants (n=229).

HIV infected (n=229)					
Variables	OI (n=55)	LTBI (n=75)	ATB (n=99)	p-values	
				ATB vs. OI	LTBI vs. ATB
Median age, years (IQR)	33 (28 to 36.5)	30.0 (27.0 to 35.1)	32.0 (27.0 to 38.2)	0.52	0.38
Median BMI, kg/m ² (IQR)	21.3 (20.0 to 24.4)	24.8 (21.3 to 30.1)	21.1 (19.0 to 24.1)	0.58	<0.001
Education, Grade (IQR)	11 (9 to 12)	10.5 (8 to 12)	10 (8.5 to 11.5)	0.25	0.78
Median CD4 (IQR)	202 (116.3-402)	393 (262 to 548)	167 (57 to 292)	0.075	<0.001
Sex, n (%)					
Male	21	19	40	0.79	0.04
Females	34	56	59		
Ethnic group					
Black	54	72	98	1.0	0.58
'Coloured'	1	2	1		
Sampling					
Apr- Sept	31	50	61	0.30	0.81
Oct - Mar	24	25	33		

3.2.2 Other infections

Table 3.3 shows the distribution of the other diseases stratified by HIV status among the OI groups. The most common OIs among the HIV uninfected individuals were cancer (40%), genitourinary disease (28%) and lower respiratory tract infection (16%). Among the HIV infected participants the most common OIs was lower respiratory tract infections (47.3%), Kaposi sarcoma (12.7 %) and gastrointestinal infection (10.9%).

Table 3.3 Number and type of opportunistic infections in the OI group

Other diseases and infections	HIV-uninfected (n=55)		HIV-infected (n=55)		Overall (n=110)	
	n	%	n	%	n	%
Opportunistic infection						
Lower respiratory tract infections (not tuberculosis)	8	16	26	47.3	34	30.9
Meningitis	0	0	5	9.1	5	4.5
Kaposi sarcoma	3	6	7	12.7	10	9.1
Liver disease	3	6	3	5.5	6	5.5
Gastrointestinal infection	1	2	6	10.9	7	6.4
Genitourinary infection	14	28	3	5.5	17	15.5
Gallstones	4	8	5	9.1	9	8.2
Cancer	22	40	0	0	22	20.0

3.2.3 Categorizing vitamin D status of study participants

Figure 3.2 shows the distribution of the vitamin D levels of the study participants. Vitamin D deficiency was highly prevalent in all the patient groups with 316 (65.8%) of the participants having 25(OH)D3 less than 50 nmol/L while 116 (23.5%) of the participants had vitamin D insufficiency (25 nmol/L < 25(OH)D3 < 75 nmol/L) and 75 (15.8%) had severe vitamin D deficiency (25(OH)D3 < 20 nmol/L).

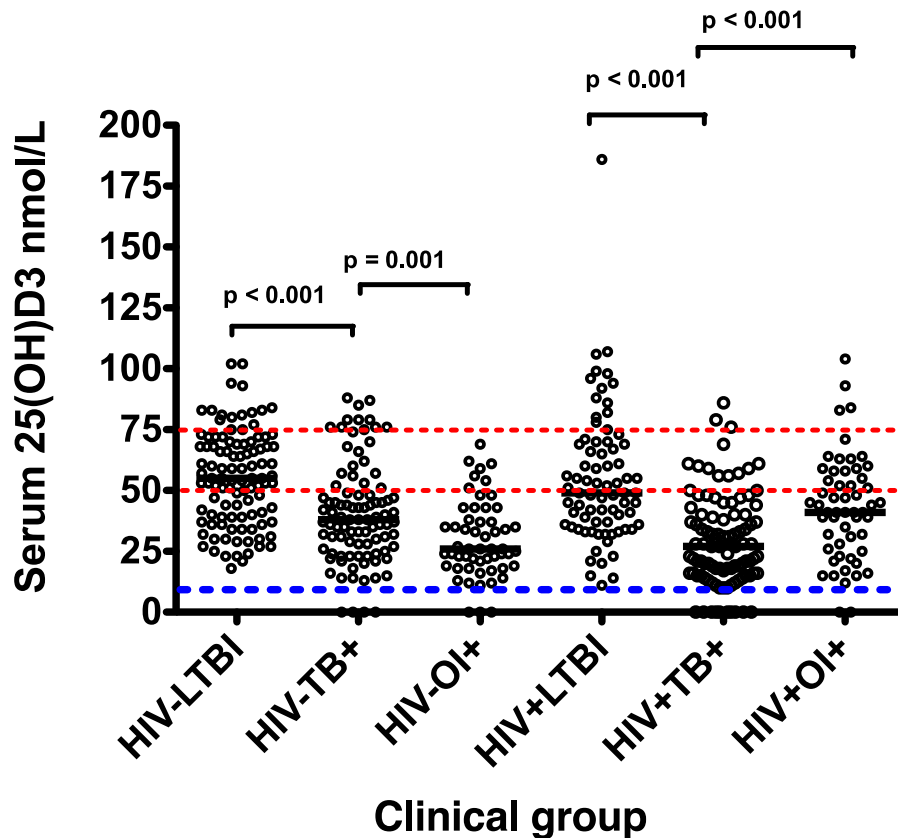


Figure 3.2 Serum 25(OH)D3 concentration stratified by HIV and TB status. Bars represent means. Blue dashed line represents limit of detection (10 nmol/L) and red dashed lines represent (50 nmol/L) and (75 nmol/L) thresholds.

Table 3.4 shows the 25(OH)D3 levels comparisons of the HIV uninfected participants. Among the HIV uninfected the comparison of ATB vs. OI shows that having an OI is associated with vitamin D levels less than 20 nmol/L (odds ratio (OR)=0.4, 95% confidence interval (95% CI): 0.2 to 0.8, $p=0.017$). In this case vitamin D deficiency was neither associated with OI nor active TB among the HIV uninfected. A diagnosis of active TB was associated with 25(OH)D3 levels below the 20 nmol/L threshold (OR=13.7, 95% CI: 1.7 to 108.2, $p<0.002$) and below the 50 nmol/L threshold (OR 5.2, 95% CI: 2.8 to 9.7, $p<0.001$) when comparing latent TB vs. active tuberculosis.

Table 3.4 Serum 25(OH)D3 concentrations of HIV uninfected study participants

HIV uninfected n=251					
Serum 25(OH)D3 threshold	OI (n=55)	LTBI (n=103)	ATB (n=93)	p values	
				ATB vs. OI p-value (Odds Ratio)	LTBI vs. ATB p value (Odds Ratio)
<20 nmol/L, n (%)	15 (27.3)	1 (1.0)	11 (11.8)	0.017 (0.4)	0.002 (13.7)
<50 nmol/L, n (%)	48 (87.3)	38 (36.9)	70 (75.3)	0.079 (0.4)	<0.001 (5.2)
<75 nmol/L, n (%)	55 (100)	87 (84.5)	82 (88.2)	0.008 (0.06)	0.45 (1.4)
50<D<75 nmol/L, n (%)	7(12.7)	48(46.6)	12 (12.9)	0.98 (1.0)	<0.001 (0.17)
	OI (n=55)	LTBI (n=103)	ATB (n=93)	ATB vs. OI	LTBI vs. ATB
Mean serum (25(OH)D3 nmol/L (s.d)	29.7 (15.8)	55.2 (19.4)	40.5 (20.8)	0.001	<0.001

Table 3.5 shows the 25(OH)D3 comparisons for the HIV infected participants. In the contrast of ATB with OI, diagnosis of active tuberculosis was associated with Vitamin D levels below the 20 nmol/L (OR=3.5, 95% CI: 1.5 to 8.2, p=0.003) and below the 50 nmol/L (OR=3.2, 95% CI: 1.45 to 7.1, p=0.003) thresholds. The assessment of LTBI vs. ATB revealed that active tuberculosis was associated with vitamin D levels beneath the 20 nmol/L (OR=14.3, 95% CI: 4.2 to 48.8, p<0.001), the 50 nmol/L (OR=5.6, 95% CI: 2.7 to 11.6, p<0.001), and beneath the 75 nmol/L (OR=7.3, 95% CI: 2.0 to 26.6, p=0.001) thresholds.

The serum 25(OH)D3 concentrations of the 480 study participants stratified by HIV and TB status are shown in Figure 3.2. For the HIV uninfected participants OI was associated with lower mean 25(OH)D3 in the comparison with ATB (mean difference 10.8 nmol/L, 95% CI: 4.3 to 17.2, p =0.001). Active tuberculosis was associated with lower mean 25(OH)D3 in the comparison with latent TB (mean difference 14.7 nmol/L, 95% CI: 9 to 20.3, p<0.001) as shown in Table 3.4.

Table 3.5 Serum 25(OH)D3 concentrations of HIV infected study participants

HIV infected n=229					
Serum 25(OH)D3 threshold	OI (n=55)	LTBI (n=75)	ATB (n=99)	p- values	
				ATB vs. OI p-value (Odds Ratio)	LTBI vs. ATB p-value (Odds Ratio)
<20 nmol/L, n (%)	8.0 (14.5)	3.0 (4.0)	37 (37.3)	0.003 (3.5)	<0.001 (14.3)
<50 nmol/L, n (%)	36 (65.5)	39 (52.0)	85 (85.9)	0.003 (3.2)	<0.001 (5.6)
<75 nmol/L, n (%)	51 (92.7)	61 (81.3)	96 (97.0)	0.226 (2.5)	0.001 (7.3)
50<D<75 nmol/L, n (%)	15 (27.3)	22 (29.3)	9 (9.1)	0.003 (0.3)	0.001 (0.2)
	OI (n=55)	LTBI (n=75)	ATB (n=99)	ATB vs. OI	LTBI vs. ATB
Mean serum (25(OH)D3 nmol/L (s.d)	43.0 (21.7)	54.7 (27.4)	28.7 (19.1)	<0.001	<0.001

In the presence of HIV infection, active TB was associated with lower mean 25(OH)D3 by comparison with either OI (mean difference 14.3 nmol/L, 95% C.I: 7.6 to 20.9, $p < 0.001$) or LTBI (mean difference 26.0 nmol/L, 95% CI: 19.1 to 33.0, $p < 0.001$).

When comparing patient groups by HIV status, HIV infection was associated with low 25(OH)D3 levels among the participants with ATB (mean difference 11.9, 95% CI: 6.2 to 17.6, $p < 0.001$). As for the participants with OI low 25(OH)D3 levels were associated with absence of HIV infection (mean difference 13.2, 95% CI: -20.4 to -6.0, $p < 0.001$). There was no significant difference of mean vitamin D levels between HIV infected and uninfected LTBI individuals (mean difference 0.51, 95%CI: -6.4 to 7.4, $p = 0.89$) as shown in Table 3.6.

Table 3.6 Mean serum 25(OH)D3 of HIV infected and uninfected clinical groups

Serum 25(OH)D threshold	HIV uninfected (Mean) (n=251)	HIV Infected (mean) (n=229)	Mean difference (95% CI)	p
ATB	40.5 (20.8)	28.7 (19.1)	11.9 (6.2 to 17.6)	<0.001
LTBI	55.2 (19.4)	54.7 (27.4)	0.51(-6.4 to 7.4)	0.89
OI	29.7 (15.8)	43.0 (21.7)	-13.2 (-20.4 to -6.0)	<0.001

3.2.4 Correlates of 25(OH)D3 Concentrations

Univariate analysis of the categorical correlates of vitamin D status showed that low vitamin D status was associated with active TB ($p<0.001$), HIV infection ($p=0.001$) and sampling between April to September inclusive ($p<0.001$). The continuous correlates showed that age and BMI were associated with vitamin D status ($p<0.001$) in both cases. There was no significant association between sex and vitamin D status as shown in Table 3.7.

There was unequal distribution of potential correlates of vitamin D deficiency such as sampling season between study groups. Therefore multivariate analysis was performed to determine potential determinants of vitamin D status independently associated with lower serum 25(OH)D3 (Table 3.7). The OI group was excluded because of its heterogeneity in the univariate and multivariate analysis models.

Lower vitamin D status was independently associated with the presence of active tuberculosis (mean difference in 25(OH)D3 concentration 28.9 nmol/L, 95% CI: 18.2 to 39.7 nmol/L, $p < 0.001$) sampling from April to September inclusive (mean difference in 25(OH)D3 concentration 10.3 nmol/L, 95% CI: 0.4 to 20.1 nmol/L, $p=0.04$) and lower BMI (mean difference in 25(OH)D3 concentration 0.50 nmol/L per unit of BMI, 95% CI: 0.06 to 0.93 nmol/L, $p=0.03$). The effect of HIV and age was lost in the multivariate model $p=0.08$ and $p=0.052$ respectively. The difference in vitamin D status between participants with and without active tuberculosis was greater in HIV-infected participants than in HIV uninfected participants (p interaction= 0.03).

Table 3.7 Correlates of vitamin D status in study participants (n = 370): univariate and multivariate analysis

n=370	Univariate analysis	Multivariate analysis		
Categorical correlates	Mean serum 25(OH) D3, nmol/L (s.d)	p-value	β (95% CI)	p-value
TB status				
Active	34.4 (20.8)	<0.001	-28.99 (-39.74 to -18.24)	<0.001
Latent	55.0 (23.0)		Ref	
HIV infection				
Yes	39.9 (26.4)	0.001	-8.30 (-17.7 to 1.10)	0.08
No	48.3 (21.3)		Ref	
Sex				
Male	41.6 (25.0)	0.06		
Female	46.5 (23.3)			
Month of sampling				
Apr-Sept	34.5 (19.8)	<0.001	-10.27 (-20.11 to -0.44)	0.04
Oct - Mar	55.3 (24.1)		Ref	
Continuous correlates	Spearman's r (95% CI)	p-value	β (95% CI)	p-value
Age, years	0.30 (-0.38 to -0.20)	<0.001	-0.25 (-0.50 to -0.00)	0.052
BMI, kg/m ²	0.21 (0.10 to 0.31)	<0.001	-0.50 (0.06 to 0.93)	0.03

3.2.5 Relationship between participant 25(OH)D3 levels and season

The seasonal variation of 25(OH)D3 levels was shown by the sinusoidal pattern of the mean 25(OH)D3 levels by month (Figure 3.3). The highest 25(OH)D3 levels were in March (mean =57.4 nmol/L, SD = 22.8) and lowest in July (mean =22.9 nmol/L, SD=18.5). Analysis by quarter showed that higher mean 25(OH)D3 levels were reached from January to March and lower from July to September inclusive (56.8 vs. 30.7 nmol/L respectively; 95% CI for difference 20.6 -31.6 nmol/L, p<0.001).

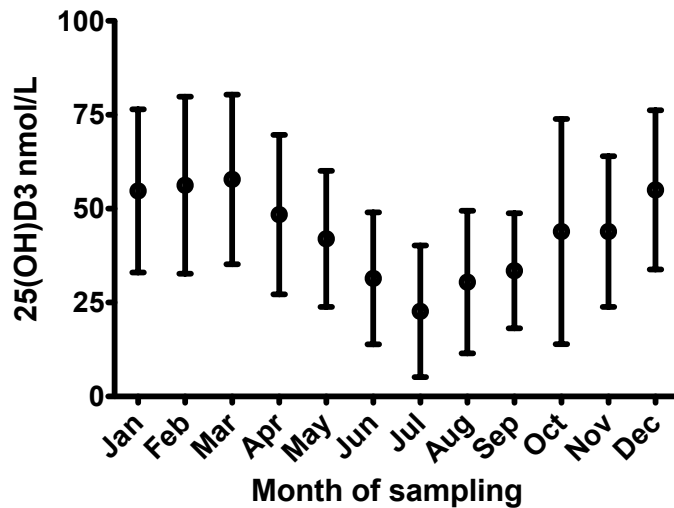


Figure 3.3 Seasonal variation in mean serum 25(OH)D3 concentration, all study participants (n = 370). Error bars indicate SD.

In addition analysis of the mean number of new tuberculosis notifications for the City of Cape Town showed consistent decrease in notifications in second quarter (April to June) of every year from 2003 to 2010 inclusive (Figure 3.4). The quarterly mean number of new tuberculosis notifications over this period was lowest in April-June and highest in October-December (4222 vs. 5080 notifications per quarter; 95% CI for difference, 642 to 1075 notifications per quarter, $p < 0.001$).

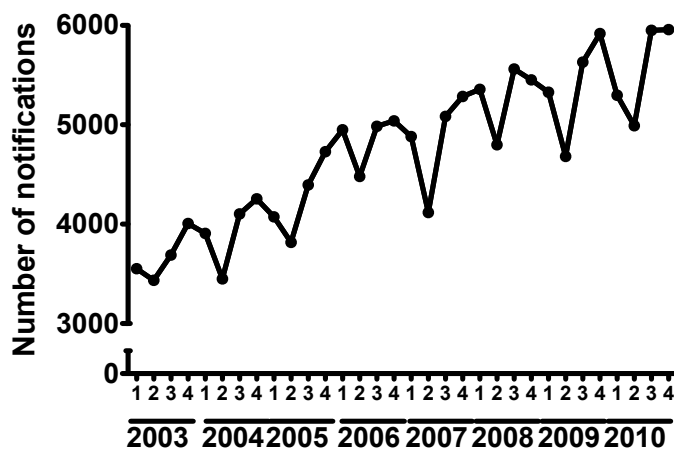


Figure 3.4 New TB notifications by quarter, City of Cape Town, 2003 to 2010.

3.2.6 Correlation between serum 25(OH)D3 levels and CD4+ cells

The determination of a relationship between serum 25(OH)D3 and CD4 count showed that there was a statistically significant weak positive correlation between serum 25(OH)D3 and CD4 cell counts among the HIV infected patients. (Spearman's correlation coefficient $r=0.24$, 95% CI 0.15 to 0.44, $p=0.002$) as shown in Figure 3.5.

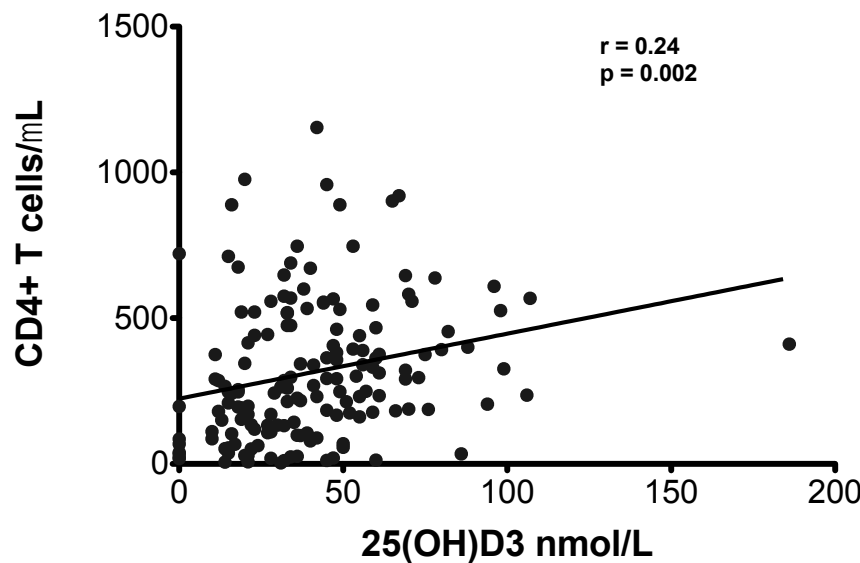


Figure 3.5 Correlation of 25(OH)D3 levels with CD4 among the HIV infected participants. (r = Spearman's correlation coefficient)

3.3 Discussion

In this cross-sectional study the association between vitamin D deficiency and tuberculosis among township dwellers was determined in Cape Town, South Africa. Vitamin D deficiency was associated with active tuberculosis in both HIV infected and HIV uninfected patients. There was a high prevalence of vitamin D deficiency in the study population. Vitamin D levels varied with season, with lowest levels of vitamin D occurring at the end of winter and highest levels in summer. In addition there was a reciprocal relationship between the seasonal variations of vitamin D levels with the tuberculosis notifications of the city of Cape Town. Active tuberculosis, sampling from April to September and BMI were independent determinants of low vitamin D levels. In addition there was a significant weak positive correlation between 25(OH)D3 levels and CD4 cell count in the patients.

The clinical and demographic characteristics of the HIV uninfected patients were generally unequally distributed between the clinical groups. Patients with OIs were significantly older than the LTBI or ATB patients, which impacts on their vitamin D status since photosynthesis of vitamin D₃ in skin decreases with increasing age due to the depletion of 7-DHC in the dermis (MacLaughlin and Holick, 1985). In addition the distribution of variables in the HIV uninfected OI group was not comparable to the other groups. There was high representation of black Africans among the HIV infected and uninfected ATB and LTBI groups who are susceptible to low 25(OH)D₃ levels because melanin limits the penetration of UVB needed for synthesis of vitamin D₃ (Clemens *et al.*, 1982).

The association of vitamin D deficiency with ATB compared to either OI or LTBI was stronger in the HIV infected compared to the HIV uninfected patients. HIV infection could be increasing the severity of vitamin D deficiency during co-infection with TB (Vescini *et al.*, 2011). The effect of HIV was further supported by the association of low vitamin D levels with ATB at the 75 nmol/L threshold in the comparison to LTBI that was not the case among the HIV uninfected. Furthermore HIV infected ATB patients had significantly low mean 25(OH)D₃ levels compared to the HIV uninfected. A high prevalence of vitamin D deficiency has been reported in HIV infected patients compared to the HIV uninfected (Villamor, 2006). The high rate of vitamin D deficiency in the TB/HIV co-infected patients might be due to chronic inflammation (Haug *et al.*, 1998a), immune activation (Vanham *et al.*, 1996) and inhibition of 1 α -hydroxylase by TNF- α through its effects on PTH metabolism (Hanevold *et al.*, 1993, Haug *et al.*, 1994, Villamor, 2006).

In contrast, vitamin D levels of HIV infected and uninfected LTBI patients were similar, this might be due to the relatively intact immune system of HIV infected LTBI shown by their fairly well-preserved median CD4 count compared to that of the HIV-infected ATB patients. 1,25(OH)₂D₃ levels have been shown to correlate with CD4 cell counts (Haug *et al.*, 1994). Higher CD4 counts and suppression of HIV viral load are associated with increased vitamin D levels (Adeyemi *et al.*, 2011). Alternatively

this might be due to absence of interaction between HIV infection and vitamin D status (Adeyemi *et al.*, 2011).

A number of studies have reported correlations between vitamin D levels with CD4 cell count (Haug *et al.*, 1994, Haug *et al.*, 1998a, Teichmann *et al.*, 2003). The significant weak positive correlation between vitamin D levels and CD4 which was noted in the HIV infected patients is in contrast to some studies, which have shown no correlation between CD4 and vitamin D levels (Madeddu *et al.*, 2004, O'Brien *et al.*, 2001).

Most of the demographic characteristics of OI group were significantly different from the LTBI and ATB patient groups since they were not ethnically matched.

These characteristics are potential confounders, which introduces bias that can compromise the results. The OI patients had a wide range of diseases most of which have been reported to be associated with depletion of 25(OH)D3 (Schwalfenberg, 2011). These include cancers, lower respiratory tract infections, liver disease (Putz-Bankuti *et al.*, 2012), gallstones (Fisher *et al.*, 2009), gastrointestinal tract and genitourinary tract infections (Schwalfenberg, 2011, Yamshchikov *et al.*, 2009). In addition those with cancer may have spent some time admitted in hospital and stayed indoors with less exposure to sunlight. The LTBI group was the best control for the study because it was composed of relatively healthy individuals with no other clinical diseases or infection.

Since this is a cross-sectional study causality cannot be inferred. It is possible that the vitamin D deficiency noted might have increased susceptibility of the patients to active tuberculosis since vitamin D insufficiency was associated with latent TB in both HIV infected and uninfected individuals in the comparison of latent TB and ATB. This might signify that they are in transition to active TB if their vitamin D levels decrease any further (Talat *et al.*, 2010, Arnedo-Pena *et al.*, 2011, Gibney *et al.*, 2008).

An alternative explanation is increased prevalence of vitamin D deficiency in TB patients could be caused by people staying indoors with closed windows thereby increasing transmission of TB or vitamin D deficiency might be as a result of tuberculosis.

The determinants of low vitamin D status in this population were active tuberculosis as well as low BMI and sampling in winter, which have been reported in other studies. *In vivo* and *in vitro* studies support the role of vitamin D in immunity against tuberculosis and more recently 1,25(OH)₂D₃ was shown to restrict the growth of both HIV and *M. tuberculosis* in co-infected macrophages (Campbell and Spector, 2012). There is a well-known inverse relationship between BMI and 25(OH)D₃ levels (Blum *et al.*, 2008). The effect of BMI is related to the fact that individuals with high BMI have higher body fat, which increases the sequestration of lipid soluble Vitamin D₃ and 25(OH)D₃ (Wortsman *et al.*, 2000).

A spectrum for the development of *M. tuberculosis* infection has been postulated (Figure 3.6) (Barry *et al.*, 2009). The inclusion of vitamin D status in the model is supported by reports of the association of vitamin D deficiency with active tuberculosis (Nnoaham and Clarke, 2008, Wilkinson *et al.*, 2000) and progression of latent tuberculosis to active tuberculosis (Gibney *et al.*, 2008, Talat *et al.*, 2010). In addition a number of studies have shown that low vitamin D levels lead to HIV disease progression (Mehta *et al.*, 2010, Viard *et al.*, 2011) with increased viral load during the course of HIV infection (Cervero *et al.*, 2012). In addition low 25(OH)D₃ levels associated with active tuberculosis result in poor treatment outcomes (Sato *et al.*, 2012).

The effect of season is related to the fact that April to September includes the winter months when there is less sunshine, which is needed for the cutaneous synthesis of vitamin D. Western Cape, South Africa has a Mediterranean climate composed of four seasons autumn starting from mid-February to April, winter May to July, Spring August to September and summer October to mid-February.

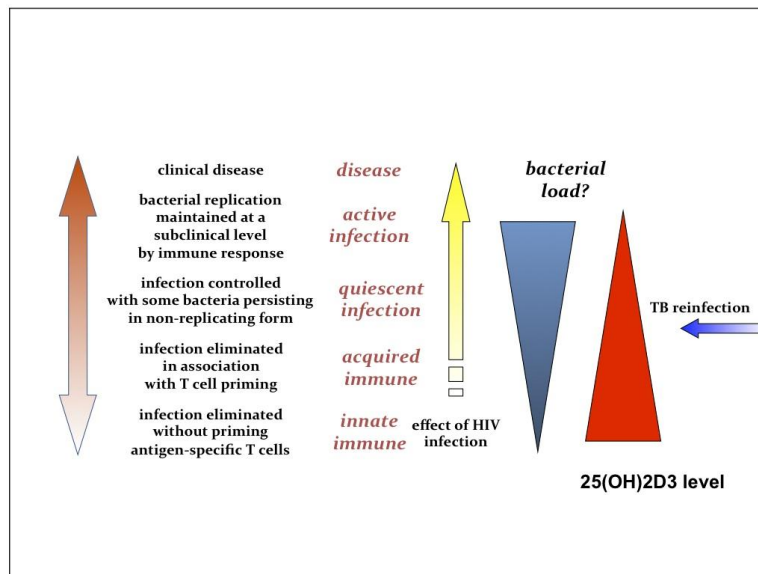


Figure 3.6 Spectrum of tuberculosis infection with vitamin D (Barry *et al.*, 2009)

The seasonal variation of Vitamin D levels which was observed is corroborated by a study carried out by Pettifor *et al.*, that demonstrated that in Cape Town low levels of vitamin D were made during winter between April and September and increased synthesis occurred in summer between October and March (Pettifor *et al.*, 1996). Seasonal variation of vitamin D levels has also been shown previously in a number of countries (Webb *et al.*, 1988, Sherman *et al.*, 1990)

Seasonal variation of vitamin D levels was linked to the seasonal variation in the tuberculosis notifications of the City of Cape Town. Increase in tuberculosis notifications in the second quarter of every year coincided with the decrease in vitamin D levels in winter. It is possible that tuberculosis is reactivated during winter due to decreased levels of vitamin D. Most patients seek medical attention when they become symptomatic usually from July at the end of winter and the beginning of spring. The results show that seasonal variation of 25(OH)D3 levels and the new tuberculosis notifications in Cape Town might be causally related.

However, the City of Cape Town tuberculosis notifications data is ecological since they are for the whole city and are not only for the participants who had their vitamin D levels measured in the study.

Similar results of seasonal variation of TB notifications that have been linked to vitamin D deficiency have previously been reported elsewhere (Luquero *et al.*, 2008, Mabaera *et al.*, 2009, Schaaf *et al.*, 1996).

The seasonal variation in tuberculosis notifications has been ascribed to seasonal variation of vitamin D bioavailability (Sita-Lumsden *et al.*, 2007), winter conditions associated with overcrowding and poor ventilation (Atun *et al.*, 2005), viral infections in winter (Luquero *et al.*, 2008) and increase in CD4+ cells and decrease in CD8+ cells during summer (Paglieroni and Holland, 1994, Rios *et al.*, 2000). The possibilities of random errors in the City of Cape Town TB notification data are negligible as the data was consistent over an 8-year period.

There are conflicting reports on the season where TB notification peaks occur in summer (Douglas *et al.*, 1996, Leung *et al.*, 2005). This might be due to differences in methods of diagnosis, healthcare systems, notification policies and seasons between countries. Santos and colleagues advocate for the application of chronobiological approaches during the development of TB control and treatment health policies (Santos *et al.*, 2012). This study supports the advocacy for supplementation with vitamin D especially during the winter seasons, which could play a role in reducing tuberculosis cases.

The study was done in Black Nguni Africans from an urban township and therefore the results can only be inferred to this group. The vitamin D results can possibly be confounded by lifestyle, diet and sun exposure; information that was not provided in the study. The OI group was heterogeneous which increases the effects of potential confounders that could affect the estimates.

There is a need for a longitudinal study, which monitors diet, exposure to sunlight and vitamin D levels in patients with latent TB and no TB infection, up until the time a diagnosis of active tuberculosis is made. This will allow the determination of the vitamin D concentration threshold at which reactivation or development of active tuberculosis on exposure to TB occurs.

The genetics of the study participants in terms of VDR, DBP and CYP27B1 polymorphism should also be determined in order to investigate their role in modulation of the immune system by $1,25(\text{OH})_2\text{D}_3$.

The data on the seasonal variation of vitamin D levels and TB notifications in Cape Town is important as it can be used in the generation of local public health policies on prevention, control and treatment of tuberculosis. This can be achieved by increasing case finding and health promotion activities during the peak seasons. These results support the need for a clinical trial of adjunctive vitamin D therapy or for prevention of tuberculosis in the high burden settings like Cape Town.

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Chapter 4: Effect of Smoking on Vitamin D Status

4.1 Literature review

Tobacco smoking is a common recreational drug, which is inhaled through the respiratory tract. Cigarette smoke contains up to 4800 constituents most of which have detrimental effects on health. The substances include poly-aromatic hydrocarbons, aromatic amines, N-nitrosamines, trace metals (Cadmium, nickel, arsenic, polonium 210), and hydroxylquinones and their effects range from being carcinogenic, toxigenic and ciliotoxic. Smoking causes a number of deleterious effects on health which include defects on bone metabolism resulting in osteoporosis, lung cancer, cardiovascular disease, emphysema, upper and lower respiratory infections (Talhout *et al.*, 2011).

There is an increasing amount of evidence supporting the importance of vitamin D in the prevention of many chronic diseases and infections that include cardiovascular diseases, diabetes mellitus, cancers, autoimmune disorders, chronic obstructive pulmonary disease (COPD) and microbial infections. Factors, which affect vitamin D sufficiency including latitude, age, exposure to sunlight and diet (Holick, 2007). Tobacco smoking has also been implicated as influencing vitamin D status (Brot *et al.*, 1999).

4.1.1 Effects of smoking on the immune system

Cigarette smoke damages the immune system through its effects on immune cells and signalling pathways (Figure 4.1). This causes defective function of the immune components resulting in increased susceptibility of smokers to microbial infections and diseases. There are conflicting results on the effect of smoking on the immune system components due to differences in models used (Sopori, 2002). Cigarette smoke activates alveolar macrophages and epithelial cells in lungs, which results in increased secretion of pro-inflammatory molecules such as reactive oxygen species (ROS) and proteolytic enzymes (Mio *et al.*, 1997, Russell *et al.*, 2002).

Increased secretion of pro-inflammatory molecules causes inflammation, which results in the oxidation of membrane lipids, DNA damage and damage of the respiratory epithelium. Cigarette smoke causes the dysfunction of immune cells such as alveoli macrophages, natural killer cells, dendritic cells, T cells and B cells. This results in the inability of the host to mount an immune response for clearance of intracellular pathogens (Stampfli and Anderson, 2009).

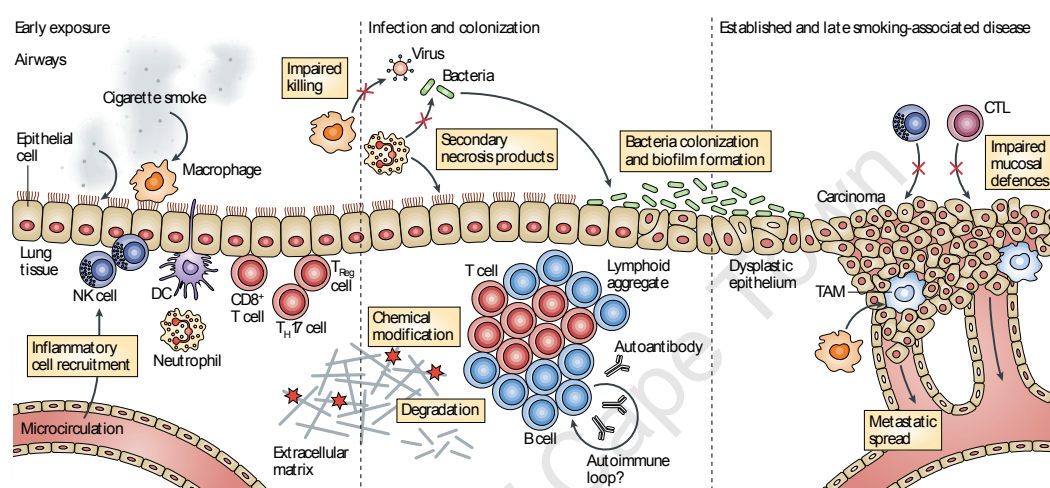


Figure 4.1 Effects of smoking in lungs (Stampfli and Anderson, 2009).

4.1.2 Effects of smoking on bone and vitamin D

Smoking cigarettes results in abnormal bone formation causing osteoporosis as it has also been shown to result in abnormal vitamin D metabolism (Brot *et al.*, 1999). Smoking inhibits calcium absorption, which is mediated by vitamin D (Hopper and Seeman, 1994).

The cigarette smoke metabolite benzo-(α)-pyrene (BaP), produced from cigarette combustion, accelerates the breakdown of 25(OH) D3 resulting in vitamin D deficiency in smokers. BaP enhances the 1,25(OH)₂D3 dependent induction of cytochrome P450 24A1 (CYP24A1) in monocytes and macrophages, which causes the depletion of 25(OH)D3 stores (Matsunawa *et al.*, 2009).

Levels of 25(OH)D₃ and intact parathyroid hormone (iPTH) have been reported to be decreased in smokers compared to non-smokers (Need *et al.*, 2002, Brot *et al.*, 1999). Effects of smoking on bone metabolism might have an impact on vitamin D levels. Calcium is essential in the synthesis of bone, vitamin D is an important mediator of bone formation and PTH is a vital hormone, which increases calcium levels by bone resorption. Smoking affects all the metabolic processes mediated by calcium, vitamin D and PTH. It has been reported to interfere with the PTH-calcium axis through inhibition of PTH secretion thereby dysregulating vitamin D metabolism and resulting in vitamin D deficiency (Hopper and Seeman, 1994, Need *et al.*, 2002, Brot *et al.*, 1999).

Vitamin D and smoking have opposing effects on the immune system. Vitamin D has anti-inflammatory effects while cigarette smoke activates the pro-inflammatory response (Stampfli and Anderson, 2009). A number of studies have shown that smokers have low 25(OH)D₃ levels compared to non-smokers (Brot *et al.*, 1999, Need *et al.*, 2002).

4.1.3 Vitamin D, smoking and respiratory disease

Smoking is associated with increased susceptibility to respiratory tract infections such as tuberculosis, pneumonia and influenza (Bates *et al.*, 2007, Arcavi and Benowitz, 2004). It is a major risk factor for chronic obstructive pulmonary disease (COPD), a disease associated with destruction of the lungs and chronic productive cough. There is increased prevalence of vitamin D deficiency among patients with COPD (Janssens *et al.*, 2011). Smoking has also been associated with increased risk of tuberculosis (Bates *et al.*, 2007). This might be related to the effects of smoking on the lung epithelium. Smoking damages the lung epithelium and causes the dysfunction of alveolar macrophages. It causes defective phagocytic function of macrophages. In addition, vitamin D deficiency might be the cause of the increased prevalence of active tuberculosis in smokers since cigarette smoke affects vitamin D metabolism (Stampfli and Anderson, 2009).

Vitamin D is very important for the maintenance of an effective innate immune system against pathogens in the lungs (Hansdottir *et al.*, 2008).

The Western Cape has the highest number of smokers in South Africa, 44.7% of men and 27% of women are smokers (Health, 2012). In view of the effects of smoking on vitamin D metabolism, bone and the immune response, I also investigated the interaction between smoking and vitamin D status in Cape Town, South Africa.

4.2 Results

4.2.1 Demographic and clinical characteristics of study participants

Among the 370 individuals in the active tuberculosis (ATB) and latent tuberculosis infection (LTBI) groups 274 with smoking information were included in the analysis and their characteristics are presented in Table 4.1. There were 59 (21.5%) current smokers, 39 (14.2%) ex-smokers and 176 (64.2%) individuals who had never smoked. Significant differences were observed for median age ($p=0.003$), median BMI ($p<0.001$), and median education ($p=0.001$) between the groups. Ex-smokers were significantly older than current smokers ($p=0.008$) or never smokers ($p=0.008$). Low BMI was associated with ex-smokers in the comparison with never smokers ($p=0.005$). Current smoking was associated with low BMI in contrast to never smokers ($p<0.001$).

There was a higher representation than expected of active tuberculosis (TB) amongst ex-smokers ($p=0.004$); HIV infection among never-smokers ($p=0.01$); sampling of ex-smokers between April to September ($p=0.03$) and a greater number of males amongst current smokers ($p<0.001$). Active TB was associated with being an ex-smoker in the comparison with either never smokers ($p=0.001$) or current smokers ($p=0.025$). HIV infection was associated with never smokers in the comparison with current smokers ($p=0.003$).

Male sex was associated with current smokers in the comparison with never smoked ($p < 0.001$), likewise male sex was associated with ex-smokers in the contrast with never smokers ($p = 0.02$). Sampling from April to September was associated with ex-smokers in the comparison with current smokers ($p = 0.01$). No significant differences were observed in the distribution of median CD4 count, median smoking years, cigarettes smoked per day and ethnicity among the smoking groups in the study.

University of Cape Town

Table 4.1 Demographic and clinical characteristics of study participants (n=274).

Variables	Current smokers (n=59)	Ex-smokers (n=39)	Never Smoked (n=176)	p
Median age, years (IQR)	31.8 (24.6 to 41.3)	32.1 (26.3 to 39.7)	26.6 (22 to 34.6)	0.003 0.08 [§] 0.08 [‡]
Median BMI, kg/m ² (IQR)	20.5 (18.6 to 22.9)	20.2 (17.7 to 24.0)	22.6 (20.3 to 25.6)	<0.001 0.005 [‡] <0.001*
Education, (IQR)	10 (8 to 11)	10 (7 to 11)	11.0 (9 to 12)	0.001
Median CD4 (IQR) In HIV infected	340 (193 to 447)	160 (98.3 to 369.8)	247.0 (100.0-408.0)	0.342
Median years smoking (IQR)	10 (4.5 to 20)	10.5 (6.5 to 18.8)		0.925
Tuberculosis n (%)				
ATB	34 (57.6)	31 (79.5)	89 (50.6)	0.004 0.025 [§] 0.001 [‡]
LTBI	25 (42.4)	8 (17.1)	87 (49.4)	
HIV status n (%)				
Yes	14 (23.7)	16 (41)	80 (45.5)	0.013 0.003*
No	45 (35.3)	23(59)	96 (54.5)	
Sex, n (%)				
Male	51(86.4)	23(59)	58(33)	<0.001 0.02 [§] 0.02 [‡] <0.001*
Females	8(13.67)	16(41)	118(67)	
Ethnic group n (%)				
Black	56 (94.9)	38(97.4)	175 (99.4)	0.075
'Coloured'	3 (5.1)	1 (2.6)	1(0.6)	
Sampling n (%)				
Apr - Sept	14 (23.7)	19 (48.7)	66 (37.7)	0.034 0.01 [§] 0.05*
Oct - Mar	45 (76.3)	20 (51.3)	109 (62.3)	
Years of smoking n(%)				
1-10	21 (52.5)	10 (50)		0.86
>10	19 (47.5)	10 (50)		
Cigarettes per day n(%)				
1-4	22 (50)	10 (52.6)		0.85
>4	22 (50)	9 (47.4)		

§ =Current smokers vs. ex-smokers, ‡= Ex-smokers vs. never smoked, *= Current smokers vs. never smoked

4.2.2 Vitamin D status of study participants

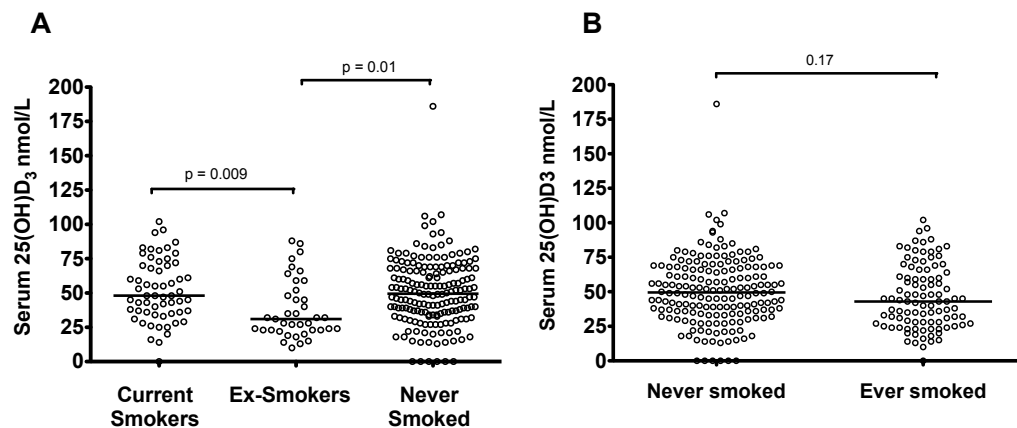


Figure 4.2 Serum 25(OH)D₃ by stratified by smoking status. (A) Divided into 3 groups (B) divided into 2 groups (ever smoked composed of current smokers and ex-smokers).

There was a significant difference between the mean vitamin D levels among the different smoking groups ($p=0.007$) (Table 4.2). Performance of post hoc tests showed that low mean vitamin D levels were associated with ex-smokers in the comparison between current smokers and ex-smokers (mean difference= 12.6, 95% CI: 2.5 to 22.8, $p=0.009$). Similarly low mean vitamin D levels were also associated with being an ex-smoker compared with the never smoked group (mean difference =14.1, 95%CI: 2.5 to 25.9, $p=0.01$) (Figure 4.2A). When the current smokers were combined with the ex-smokers to make the ever smoked group, there was no significant difference in mean vitamin D levels between this group and the never smoked group (mean difference = -4.14, 95% CI: -10.1 to 1.9, $p=0.17$) (Figure 4.2B).

A high prevalence of vitamin D deficiency was observed in the study population with 54% classified as vitamin D deficient, 30.7% were vitamin D insufficient and 15.3% were vitamin D sufficient. There was no association between vitamin D status at the 20nmol/L and 75nmol/L thresholds with smoking status. Analysis of the vitamin D status at the 50nmol/L threshold revealed that vitamin D deficiency was associated with smoking status ($p<0.02$) (Table 4.2).

Table 4.2 Serum 25(OH)D3 concentrations of study participants stratified by smoking status (n=274).

Serum 25(OH)D3 threshold	Current Smokers (n=59)	Ex-smokers (n=39)	Never smoked (n=176)	p-value (chi-square test)
<20 nmol/L, n (%)	3 (5.1)	6 (15.4)	16 (9.1)	0.22
<50 nmol/L, n (%)	31 (52.5)	29 (74.4)	88 (50)	0.02
<75 nmol/L, n (%)	47 (79.7)	35 (89.7)	150 (85.2)	0.38
	Current Smokers (n=59)	Ex-smokers (n=39)	Never smoked (n=176)	p-value (One-way ANOVA)
Mean serum 25(OH)D3 nmol/L (s.d)	52.0 (22.5)	37.9 (21.5)	50.5 (24.7)	0.007

Further analysis at the 50 nmol/L threshold demonstrated that vitamin D deficiency was associated with being an ex-smoker when compared with current smokers (odds ratio (OR)= 0.16, 95% confidence interval (CI): 0.03 to 0.92, p=0.03). In contrast, the comparison between having never smoked and ex-smokers also showed that vitamin D deficiency was associated with being an ex-smoker (OR= 2.9, 95% CI: 1.3 to 6.3, p=0.006). There was no association with vitamin D deficiency in the comparison between ex-smokers and current smokers (p=1) (Table 4.3).

Table 4.3 Post hoc analysis of vitamin D deficiency and mean vitamin D levels of smoking groups (n=274).

Serum 25(OH)D3 threshold	Current Smokers (n=59)	Ex-smokers (n=39)	Never smokers (n=176)	p-values		
				Current Smokers vs. Ex smokers	Ex-smokers vs. Never smoke	Current Smokers vs. Never smoked
<50 nmol/L, n (%)	31 (52.5)	29 (74.4)	88 (50)	0.03	0.006	0.74
	Current Smokers (n=59)	Ex-smokers (n=39)	Never smoked (n=176)			
Mean 25(OH)D3 nmol/L (s.d)	52.0 (22.5)	37.9 (21.5)	50.5 (24.7)	0.009	0.01	1

4.2.3 Analysis of risk factors of vitamin D status

Univariate analysis of the possible categorical determinants of vitamin D status indicated that low vitamin D status was associated with active tuberculosis ($p=0.01$), male sex ($p=0.04$), sampling between April and September inclusive ($p < 0.001$), and smoking status of being an ex-smoker ($p=0.007$). For the continuous correlates vitamin D status significantly correlated with age ($p < 0.001$) and BMI ($p < 0.001$), (Table 4.4).

Multivariate analysis indicated that low vitamin D status was independently associated with active tuberculosis ($p=0.001$), month of sampling ($p=0.001$) and BMI ($p=0.02$) (Table 4.4). In addition multivariate analysis showed that low BMI was independently associated with being an ex-smoker ($p=0.02$), active tuberculosis ($p=0.04$) and male sex ($p < 0.001$) (Table 4.5).

Table 4.4 Correlates of vitamin D status: univariate and multivariate analysis (n=274).

	Univariate analysis	Multivariate analysis		
Categorical correlates	Mean serum 25(OH) D, nmol/L (s.d)	p-value	β (95% CI)	p-value
TB status				
Active	38.5 (20.3)	<0.001	-17.6 (-27.8 to 7.3)	0.001
Latent	62.6 (23.0)		Ref	
HIV infection				
Yes	47.1 (26.9)	0.27		
No	50.4 (22.2)			
Sex				
Male	45.9 (24.5)	0.04	5.4 (-5.5 to 16.4)	0.33
Female	51.9 (23.6)		Ref	
Month of sampling				
Apr-Sept	37.7 (20.2)	<0.001	-18.0 (-28.6 to -7.4)	0.001
Oct - Mar	55.5 (24.0)		Ref	
Smoking status				
Current smokers	51.98 (22.54)	0.007	5.0 (-15.6 to 25.6)	0.63
Ex-smokers	37.90 (21.52)		-3.4 (-24.2 to 17.3)	0.75
Never Smoked	50.52 (24.71)		Ref	
Continuous correlates	Spearman's r (95% CI)	p-value	β (95% CI)	p-value
Age, years	-0.33 (-0.83 to -0.31)	<0.001	-0.15 (-0.43 to 0.14)	0.31
BMI, kg/m ²	0.28 (0.73 to 0.19)	<0.001	0.76 (0.11 to 1.40)	0.02

Table 4.5 Correlates of BMI: univariate and multivariate analysis (n=274)

	Univariate analysis	Multivariate analysis		
Categorical correlates	Mean BMI, kg/m ² (s.d)	p-value	β (95% CI)	p-value
TB status				
Active	21.4 (4.3)	<0.001	1.6 (0.06 to 3.2)	0.04
Latent	24.3 (4.6)		Ref	
HIV infection				
Yes	47.1 (26.9)	0.27		
No	50.4 (22.2)			
Sex				
Male	20.7 (3.4)	<0.001	-4.0 (-5.9 to -2.1)	<0.001
Female	24.6 (5.0)		Ref	
Month of sampling				
Apr-Sept	22.4 (4.2)	<0.42		
Oct - Mar	22.9 (5.0)			
Smoking status				
Current smokers	21.0 (3.4)	<0.001	-1.4 (-7.3 to 4.5)	0.63
Ex-smokers	21.4 (4.7)		-3.5 (-6.5 to -0.6)	0.02
Never Smoked	23.6 (4.9)		Ref	
Continuous correlates	Spearman's r (95% CI)	p-value	β (95% CI)	p-value
Age, years	-0.072 (- 0.078 to 3.24)	0.25		
Education	-0.2 (- 0.187 to 0.52)	0.001	-0.09 (-3.2 to 0.1)	0.40

4.3 Discussion

This analysis showed that low mean vitamin D levels and vitamin D deficiency were associated with ex-smokers in the univariate analysis, but in the multivariate analysis smoking was not independently associated with low vitamin D status. Just like the earlier analysis in chapter 3 active tuberculosis, sampling from April to September and BMI were independently associated with low vitamin D levels among the study participants.

Low BMI was independently associated with active tuberculosis, male sex and being an ex-smoker. Association of smoking with low BMI is a common feature in individuals who smoke worldwide (Molarius *et al.*, 1997). Active tuberculosis was associated with participants who were ex-smokers. A large number of epidemiological studies show that smoking is a major risk factor for active tuberculosis and increases the risk of progression from latent tuberculosis infection to active disease among current smokers and ex-smokers compared to those that have never smoked (Bates *et al.*, 2007). The increased susceptibility to TB is mainly dependent on the number of years spend smoking (Lin HH *et al.*, 2009, Bates *et al.*, 2007).

The biological mechanism by which smoking increases respiratory infections is by suppressing the innate and adaptive immune responses resulting in decreased secretion of pro-inflammatory cytokines, immunoglobulins, mechanical disruption of cilia function, defective function of alveoli macrophages, natural killer cells, and dendritic cells in response to infection (Arcavi and Benowitz, 2004, Stampfli and Anderson, 2009). In this study active tuberculosis was associated with being an ex-smoker. This might be explained by the fact that ex-smokers were significantly older than current smokers and as smokers get older many are usually compelled by health problems to stop.

A significantly large number of participants that have never smoked were infected with HIV. This was contrary to a number of reports that found smokers to be at increased risk of HIV infection than never smokers even though there is no plausible biological evidence of mechanism that support the high risk (Furber *et al.*, 2007). Male sex was also associated with being a current smoker in comparison with ex-smokers or never smoked. This is in line with studies elsewhere in which generally there is higher prevalence of smokers among males compared to females (Bolego *et al.*, 2002).

Smoking was not an independent determinant of low 25(OH)D3 levels. The association of vitamin D deficiency levels with ex-smokers in the univariate analysis might be due to confounding results. There are conflicting results on the effect of smoking on vitamin D status between smokers and non-smokers with some studies reporting lower levels in smokers (Hermann *et al.*, 2000, Brot *et al.*, 1999, Lorentzon *et al.*, 2007, Szulc *et al.*, 2002) while others report no significant difference in results between smokers and non-smokers (Arunabh *et al.*, 2003, Need *et al.*, 2002, Scragg *et al.*, 1992). The variation of results might be due to the use of different methods of vitamin D measurement. Electrochemiluminescence methods result in higher vitamin D levels in smokers than in non-smokers due to interference by smoke molecules in samples (Grimnes *et al.*, 2010). In addition, the conflicting results might be due to heterogeneity among the different populations as they vary in genetics, amount of smoking and nutrition (Sopori, 2002).

In this study, ex-smokers were more susceptible to vitamin D deficiency, while other studies observed this in current smokers. This could be caused by the older age of the ex-smokers in this study, which makes them more susceptible to vitamin D deficiency. Similarly, a decrease in vitamin D levels in ex-smokers after stopping for at least a month has been reported in men (Supervia *et al.*, 2006). This could be due to the irreversible effects of smoking on the vitamin D –PTH system resulting in maintenance of low PTH and vitamin D levels (Need *et al.*, 2002, Brot *et al.*, 1999, Supervia *et al.*, 2006). After cessation of cigarette smoking some of its pathological effects and damage is maintained for many years. These include damaged tissues

and elevation of inflammatory molecules like CRP, TNF- α , IL-6, fibrinogen and white blood cell count which makes the high susceptibility to cancer and respiratory tract infections persist in ex-smokers even after stopping smoking (Wannamethee *et al.*, 2005, Helmersson *et al.*, 2005, Bermudez *et al.*, 2002, Costenbader and Karlson, 2006). In this case it might have resulted in ex-smokers still being at high risk of vitamin D deficiency as systemic inflammation can result in inhibition of PTH by the pro-inflammatory mediators such as TNF- α (Haug *et al.*, 1994, Haug *et al.*, 1998b).

Low vitamin D levels were also associated with male sex whereas other studies have reported association between low vitamin D levels with female sex (Hyppönen and Power, 2007, Levis *et al.*, 2005, Meddeb *et al.*, 2005). The difference can be explained by the fact that smoking was associated with male sex while on the other hand smoking has been associated with vitamin D deficiency. Cigarette smoking is suggested to be a possible determinant of sex differences for the prevalence of active tuberculosis (Watkins and Plant, 2006).

The limitations of this study were that it was a cross-sectional study and therefore causality cannot be assigned. The self-reporting of smoking status could have possibly introduced bias owing to effects of social desirability from the participants and may underestimate the prevalence of smoking, particularly among active tuberculosis patients. Information on the brands of cigarettes and method of smoking was not obtained this is important for accessing the degree of exposure as the smoke composition of different brands of cigarettes vary. Ninety-six participants were not included in this analysis as smoking information was absent. Participants with smoking data had significantly lower BMI, were mostly males, HIV infected and were sampled during summer (October to March). They also had higher vitamin D levels, which can affect the generalizability of the results.

Smoking was not an independent determinant of vitamin D status. The effect of cigarette smoking noted during the univariate analysis might potentially be due to confounding.

Active tuberculosis, sampling from April to September and low BMI were maintained as potential risk factors of low vitamin D levels in this population setting. Cigarette smoking was eliminated as a potential confounder of the association between vitamin D deficiency with active tuberculosis. Therefore since active tuberculosis and sampling during winter have also been reported globally as potential risk factors of low vitamin D levels their association could be correct.

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Chapter 5: Immunophenotyping Of Mφ1 and Mφ2

5.1 Literature review

5.1.1 The mononuclear phagocyte system

The mononuclear phagocyte system that was postulated in 1972 consists of committed monocyte myeloid precursors, monocytes, tissue macrophages and dendritic cells. The mononuclear phagocytes are classified according to their morphology, function, haemopoietic origin and kinetics (van Furth *et al.*, 1972). The study of the biology of cells in mononuclear phagocyte system is important for understanding susceptibility and control to infectious diseases and inflammatory responses.

Macrophages are generated from haemopoietic stem cells in the bone marrow, which proliferate and differentiate to monocytes that leave BM into peripheral blood circulation. Monocytes can spend up to 4 days in the peripheral blood circulation before they move into inflammatory sites in response to chemokines. On stimulation with various cytokines, chemokines and tissue factors monocytes attach through selectin and integrin adhesion molecules to blood vessel wall endothelium and extravasate into tissues where they mature into tissue macrophages (Penna *et al.*, 2002). The monocytes continuously enter tissues even in the absence of inflammation where they differentiate into macrophages and those that do not migrate into tissues undergo apoptosis (Mangan and Wahl, 1991).

Apart from the extravasation of monocytes, tissue macrophages are also replenished by local proliferation of tissue macrophages due to the effects of cytokines such as monocyte-colony stimulating factor (M-CSF), IL-3 and IL-4 independent of the bone marrow (Jenkins *et al.*, 2011).

Monocytes mature into specific types of macrophages depending on their tissue localization: alveolar macrophages in lungs, Kupffer cells in liver, Langerhans cells in skin, osteoclasts in bone, microglial cells in brain, peritoneal macrophages in abdominal cavity, synovial A cells in joint synovial fluid and mesengial cells in kidneys (Gordon and Taylor, 2005). In addition tissue macrophages are found as both fixed and free: free macrophages are found in pleural, synovial, alveolar spaces and peritoneal while the fixed ones are found in liver Kupffer cells, bone osteoclasts, splenic macrophages and brain microglia. Macrophages are located at strategic positions in tissues where they encounter invading pathogens or host components, which need to be phagocytosed.

Osteoclasts develop from peripheral blood when they are exposed to a microenvironments composed of M-CSF and receptor activator of nuclear factor-kappa β ligand (RANKL) from stromal cells (Husheem *et al.*, 2005). Also, peritoneal macrophages are generated in abdominal tissues in the presence of M-CSF (Xu *et al.*, 2007) and alveolar macrophages are generated from monocytes in presents of granulocyte-macrophage colony stimulating factor (GM-CSF) and surfactant protein A and D (SP-A) and (SP-D) (Guth *et al.*, 2009). Furthermore, microglia cells are generated from monocytes in the presents of GM-CSF and IL-3 (Kloss *et al.*, 1997).

5.1.2 Roles for macrophages in immunity

Macrophages have many different functions that are dependent on their ability to change their phenotype and properties depending on the microenvironment (Gordon and Taylor, 2005, Stout and Suttles, 2004). They are very important mediators of many biological processes such as immunity against infection and malignancy, healing and clearance of cellular debris (Gordon, 2003).

Despite the ability of macrophages to efficiently detect and phagocytose microbes, some facultative intracellular pathogens are able to use them to avoid detection and for transport to target tissues.

Infection of macrophages with *M. tuberculosis* results in inhibition of fusion of the phagosome and lysosome allowing their survival inside the cells. In addition HIV uses host macrophages as a reservoir of infection and also use them to gain accessibility to the central nervous system tissues.

The typical macrophages receptors CD11b an integrin mediates cell-to-cell interaction and phagocytosis of iC3b opsonized pathogens or cells while CD33 a sialoadhesin and CD15, mediate cell-cell interaction and signalling. In addition they express CD14 which is involved in recognition and antimicrobial response against lipopolysaccharide binding protein opsonized microbial components (Schumann *et al.*, 1996) and CD16 a low affinity Fc receptor involved in recognition, phagocytosis of antibody opsonized molecules and antibody dependent cell cytotoxicity (ADCC) (Keler *et al.*, 2000). Macrophages phagocytose microbes or antigens, process them and present them to T cells via their major histocompatibility complex (MHC) Class II molecules. They are able to influence the type of T cell and B cell response to be stimulated by secretion of different cytokines e.g. IL-12 and IL-10, which stimulate activation of Th1 CD4 T lymphocytes and Th2 CD4 T lymphocytes respectively (Verreck *et al.*, 2004).

5.1.3 Macrophage polarization and activation

Macrophage classification in the mononuclear phagocyte system is complex due to the fact that the cells continuously change depending on their microenvironment resulting in changes in their functional pattern characteristics. There are many different types of macrophage activation states, which are a continuum from pro-inflammatory to anti-inflammatory cells with no clear separation (Mantovani *et al.*, 2004). The three main types of macrophage polarised states, are innate activation, classical activation and alternative activation (Gordon, 2003). However another proposal is that macrophages should be classified as classical activated macrophages, wound healing macrophages and regulatory macrophages (Mosser and Edwards, 2008).

The high plasticity and heterogeneity of macrophages means they readily respond to environmental signals in their milieu. The significance of this *in vivo* is that there is a spectrum of macrophage activation continuum which is important for the regulation of the immune response as the ability of the cells to respond to the changes in cytokines, microbes and host debris materials allows the cells not to be over stimulated. It allows the activation of the cells to secrete pro-inflammatory and antimicrobial molecules in the presence of pathogens and the return to the steady state after the clearance or prevent over stimulation by production of anti-inflammatory molecules IL-4, IL-10 and $1,25(\text{OH})_2\text{D}_3$, which change the phenotype of the cells towards alternative activation. It seems the macrophages are able to respond to all cytokines and molecules in their milieu by being able to be refractory to others and being stimulated by others depending on the concentration such that under steady state all the different phenotypes might be present with the levels being skewed when the balance is disturbed (Mantovani *et al.*, 2004, Gordon, 2003).

5.1.3.1 Innate activation

The stimulation of macrophage Toll like receptors (TLR) by microbial molecules such as lipopolysaccharide (LPS) results in the innate activation of the cells. The innate activated cells have high surface expression of co-stimulatory molecules such as HLA-DR, CD86 that allow them to effectively stimulate CD4⁺ T cells. The innate activated macrophages secrete pro-inflammatory cytokines like interferon α and β , which stimulate expression of inducible nitric oxide synthase (iNOS) and production of reactive oxygen species (ROS) and nitric oxide (NO). They are efficient phagocytes due to increased expression of scavenger receptors and mannose receptors (Nau *et al.*, 2002, Gordon, 2003).

5.1.3.2 Classical activation

Stimulation of macrophages with Interferon- γ in the presence or absence of LPS results in a pro-inflammatory type of macrophages named classical activated macrophages (M1) (Gordon, 2003).

The M1 macrophages secrete high levels of pro-inflammatory cytokines and chemokines: IL-12, IL-6, IL-1 β , TNF- α , IP-10, macrophage inhibitory peptide-1 α (MIP-1 α), macrophage chemotactic protein-1 (MCP-1) and low levels of IL-10 (Mantovani *et al.*, 2004, Mosser, 2003). They have increased expression of MHC-II, CD86, CD14, CCR5 and down regulation of mannose receptors (Mosser, 2003, Mantovani *et al.*, 2004). M1 macrophages have increased antimicrobial activity and are highly effective in killing of intracellular pathogens and tumour resistance. In general M1 macrophages are associated with type 1 inflammation, delayed type hypersensitivity (DTH), and Th1 immune response (Mantovani *et al.*, 2002).

5.1.3.3 Alternative activation

Alternatively activated macrophages are generated by stimulation of macrophages with a variety of stimulatory molecules like IL-4, IL-13, IL-10, glucocorticoid and 1,25(OH) $_2$ D3 (Goerdts and Orfanos, 1999). Three subclasses of alternatively activated macrophages have been proposed; stimulation of the macrophages with IL-4 or IL-13 results in generation of M2a macrophages, stimulation with immune complexes and agonists of TLR or IL-1R results in generation of M2b macrophages, and M2c macrophages are generated on stimulation of the macrophages with IL-10, glucocorticoids and 1,25(OH) $_2$ D3 (Mantovani *et al.*, 2004, Gordon, 2003)

The alternatively activated M2a macrophages express MHC class II receptors, mannose receptors and scavenger receptors, which they use for detection and phagocytosis of the materials. The M2a cells are mainly involved in mediation of Th2 immune responses, type II inflammation, allergy, tissue repair, collagen synthesis and encapsulation of parasites.

The M2b and M2c activated macrophages are mainly involved in immune regulation. The M2b activated macrophages mediate Th2 immune responses and immune regulation.

They are characterized with surface expression of co-stimulatory molecules MHC-II and CD86 for stimulation of CD4⁺ T cells. On stimulation, they secrete increased levels of IL-10, TGF- β TNF- α , IL-1 and IL-6 and low levels of IL-12 (Mantovani *et al.*, 2004).

5.1.4 *In vitro* generation of macrophages

Human monocytic leukaemia cell lines such as Mono Mac-1, Mac- 6, THP-1, HL-60 and U937 at different stages of differentiation can be used as macrophage model since primary tissue macrophages cannot be readily extracted and proliferated *ex vivo*. Stimulation of these monocytic cell lines with phorbol myristate acetate (PMA) or 1,25(OH)₂D₃ results in their differentiation to macrophages like cells. The phenotype of the macrophages varies depending on the duration and type of stimulation (Kohro *et al.*, 2004). These cell lines are established from disordered tissues and continuous passaging in the laboratory may also results in alteration of their genomic constitutions and characteristics (Odero *et al.*, 2000, Abrink *et al.*, 1994). However some of the advantages of cell line use is their high accessibility, ease of standardization and unlimited expansion.

Monocyte derived macrophages (MDM) reflect better the characteristics of tissue macrophages. They are generated from monocytes *in vitro* by stimulation with M-CSF, GM-CSF, Vitamin D, IL-3 or adherence (Daigneault *et al.*, 2010, Geissler *et al.*, 1989). The phenotypic characteristics, effector functions and morphology of the monocyte-derived macrophages generated in presence of these cytokines are different. GM-CSF and M-CSF concentrations ranging from 5 ng/ml to 100 ng/ml have been used for the generation of monocyte-derived macrophages in the literature. There is no defined standard concentration of the cytokines, used for the generation of the MDMs by researchers, which results in researchers generating cells with different phenotypes and morphology from use of the same cytokines, which may present problems when comparing results.

M-CSF is a disulphide linked homodimeric growth factor that acts on monocytes, macrophages and dendritic cells via the MCSFR receptor (CD115) (Sherr *et al.*, 1985). It controls the differentiation, proliferation, and maturation of blood cells in the mononuclear phagocytic lineage from pluripotent stem cells residing in the bone marrow and the differentiation of monocytes to macrophages (Irvine *et al.*, 2008). M-CSF is the only primary macrophage growth factor, which is detectable in peripheral blood under steady-state conditions (Bartocci *et al.*, 1987).

In vivo levels of M-CSF are present in normal serum around 30ng/ml (Irvine *et al.*, 2008) and favours an M2 polarized phenotype during human to macrophage differentiation (Verreck *et al.*, 2004, Martinez *et al.*, 2009). M-CSF is continuously secreted by cells that include fibroblasts, macrophages, endothelial cells and osteoblasts. Monocytes generated via adherence only are also differentiated by M-CSF as the adherence of the cells to plastic induces the monocytes to produce autocrine M-CSF and TNF (Komuro *et al.*, 2005).

GM-CSF is a haemopoietic growth factor and pro-inflammatory cytokine that is involved in inflammation, activation, proliferation, differentiation and recruitment of macrophages and granulocytes (Hamilton, 2002). It activates the effector functions of mononuclear phagocytes and granulocytes. It is produced by a variety of immune cells, which include T cells, B cells, macrophages, fibroblasts, mast cells and endothelial cells in response to various stimuli. GM-CSF enhances oxidative metabolism, antibody dependent cell cytotoxicity (ADCC), microbial phagocytosis, superoxide production and tumoricidal activity of the macrophages (Cannistra *et al.*, 1988). GM-CSF in particular is important in the development of alveolar macrophages as its deficiency results in defective alveolar function and alveoli proteinosis (Stanley *et al.*, 1994) and deficiency of M-CSF results in reduced monocytes and macrophages in the body (Schonlau *et al.*, 2003). GM-CSF is a pro-inflammatory cytokine that is not detectable in circulation and is found locally at sites of inflammation (Hamilton, 2002).

In general monocyte derived macrophages generated in the presence of GM-CSF are pro-inflammatory secreting large amounts of IL-12, TNF- α , IL-1, IL-6, IL-23 and low levels of IL-10. The M-CSF generated macrophages are anti-inflammatory secreting high levels of IL-10 and IL-4 while inhibiting IL-12 and IL-6 secretion (Verreck *et al.*, 2004, Verreck *et al.*, 2006). GM-CSF generated macrophages have the M1 or M1 like phenotype (M ϕ 1) while the M-CSF generated macrophages are said to have an M2 like phenotype (M ϕ 2) (Hamilton, 2008, Fleetwood *et al.*, 2009, Verreck *et al.*, 2004).

The two MDM populations have distinct morphological characteristics. M ϕ 1 are round with a centrally or eccentrically placed nucleus and M ϕ 2 have a fusiform or spindle shaped structure. Both M ϕ 1 and M ϕ 2 have been reported to have surface expression of CD11b, CD11c, CD14, CD4, CCR5, CXCR4, Fc γ receptors and HLADR (Young *et al.*, 1990, Metcalf *et al.*, 1986). In addition M ϕ 1 express CD71, HLADQ and which are not expressed by M ϕ 2. Furthermore M ϕ 2 secrete large amounts of hydrogen peroxide and express integrin receptor $\alpha\beta$ 5 whereas M ϕ 1 express $\alpha\beta$ 3 integrin and secrete less hydrogen peroxide due to their high catalase expression (De Nichilo and Burns, 1993, Akagawa, 2002, Komuro *et al.*, 2001).

In terms of susceptibility to infectious diseases M ϕ 2 are more susceptible to HIV infection and resistant to *M. tuberculosis* infection while M ϕ 1 are resistant to HIV infection and more susceptible to *M. tuberculosis* infection (Akagawa, 2002, Matsuda *et al.*, 1995). It is thought that HIV replication is inhibited post transcriptionally since both M ϕ 1 and M ϕ 2 express the same amount viral DNA (Matsuda *et al.*, 1995). Also M ϕ 2 macrophages express the natural resistance associated macrophage protein 1 (NRAMP1) gene while M ϕ 1 do not express it. The NRAMP1 gene is associated with increased resistance to *M. tuberculosis* infection in humans (Bellamy *et al.*, 1998). Moreover M ϕ 2 suppresses IFN- γ production by lymphocytes in response to purified protein derivative (PPD) while M ϕ 1 does not (Mochida-Nishimura *et al.*, 2001).

Gene expression studies by Brocheriou and colleagues demonstrated that M ϕ 2 had low expression of CD163L and increased expression of stabilin-1 (STAB1) mediator of tissue repair and modelling, selenoprotein-1 (SEPP1) an anti-oxidant selenoprotein and a disintegrin and metalloproteinase domain-like protein decysin-1 (ADAMDEC1) that are anti-inflammatory. The M ϕ 1 had increased expression of pro-inflammatory genes CD163 molecule like-1 (CD163L), pro-platelet basic protein (PPBP) a chemokine and activator of neutrophils and arachidonate 5-lipoxygenase-activating protein (ALOX5AP) that encodes a 5-lipoxygenase involved in leukotriene synthesis (Brochérou *et al.*, 2011). Stimulation of M ϕ 1 with M-CSF results in decreased secretion of anti-inflammatory cytokines with the phenotype moving towards the anti-inflammatory (Fleetwood *et al.*, 2007). The macrophages can also be differentiated by their surface marker expression.

All this data show that the GM-CSF and M-CSF differentiated macrophages differ in their characteristics and the level of polarization. The M ϕ 1 have a pro-inflammatory phenotype while the M ϕ 2 macrophages have an anti-inflammatory phenotype. Therefore we sought to identify the phenotype and cell surface markers that are differentially expressed by macrophages generated from use of GM-CSF (M ϕ 1) and M-CSF (M ϕ 2) that were to be used for the macrophage model of HIV and *M. tuberculosis* co-infection.

5.2 Results

5.2.1 Morphological characteristics of M ϕ 1 and M ϕ 2

M ϕ 1 and M ϕ 2 were generated in parallel from peripheral blood CD14⁺ monocytes derived from same donor in the presence of GM-CSF and M-CSF respectively. After 6 days of differentiation M ϕ 1 were homogenously large round cells with an eccentrically placed nucleus “fried egg” morphology (Figure 5.1A). In contrast the M ϕ 2 were mainly composed of irregularly round cells with a few spindle or fibroblastic cells (Figure 5.1B).

A

B

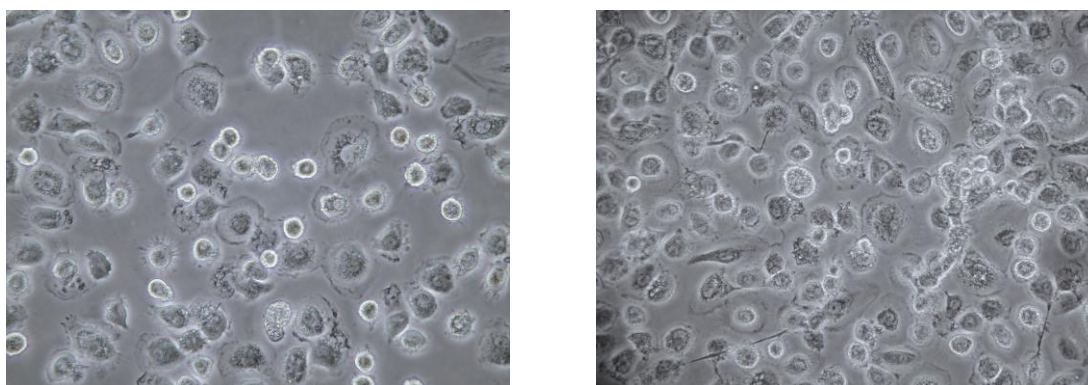


Figure 5.1 Effects of CSFs on differentiation of monocytes to macrophages (A) M ϕ 1 (B) M ϕ 2

5.2.2 Analysis of MDM by flow cytometry

5.2.2.1 Gating strategy

To identify macrophages, forward scatter (FSC) vs. side scatter (SSC) was used to determine the distribution of the monocyte-derived macrophages (MDM) based on size and intracellular granularity. The first macrophage gate was set in a FSC vs. SSC dot plot and applied to a SSC vs. CD11b dot plot to set the macrophage gate around the CD11b positive cells as shown in Figure 5.2. These CD11b positive monocyte derived macrophages (MDMs) were analysed for various surface receptor expression.

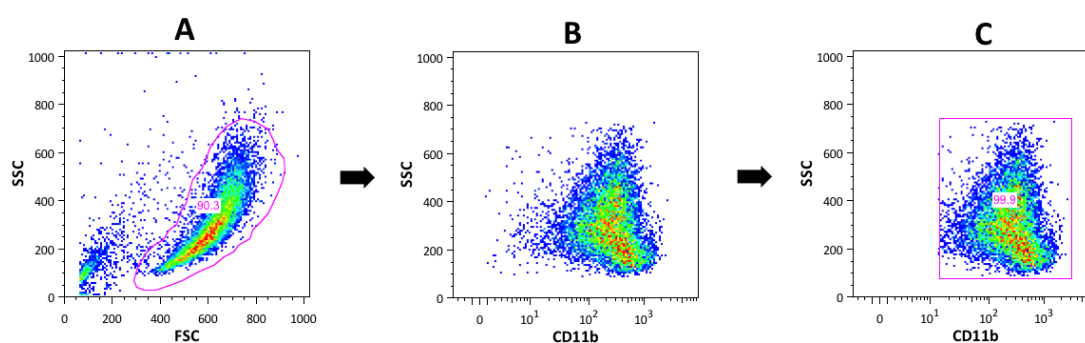


Figure 5.2. The gating strategy: macrophages were identified by staining for CD11b. First gate was set in a FSC vs. SSC dot plot (A) and applied to a CD11b vs. SSC dot plot (B) to set the macrophage gate around the CD11b positive cells (C).

Figure 5.3A shows the forward scatter vs side scatter density plots of M ϕ 1 and M ϕ 2. The subpopulation consisting of cells with high FSC and SSC gate G1 are the monocyte derived macrophages. The percentage of CD11b positive cells on gating of this population did not differ between the M ϕ 1 and M ϕ 2 ($99.3\pm0.5\%$ and $99.3\pm0.94\%$, respectively) (Table 5.1). The dot plots show results of a representative donor shown in Figure 5.3 B to F.

Representative histograms for CD11b, TLR2, CD33, CD14, CD16, CD15, HLADR and DC-SIGN of the M ϕ 1 and M ϕ 2 are shown in Figure 5.4.

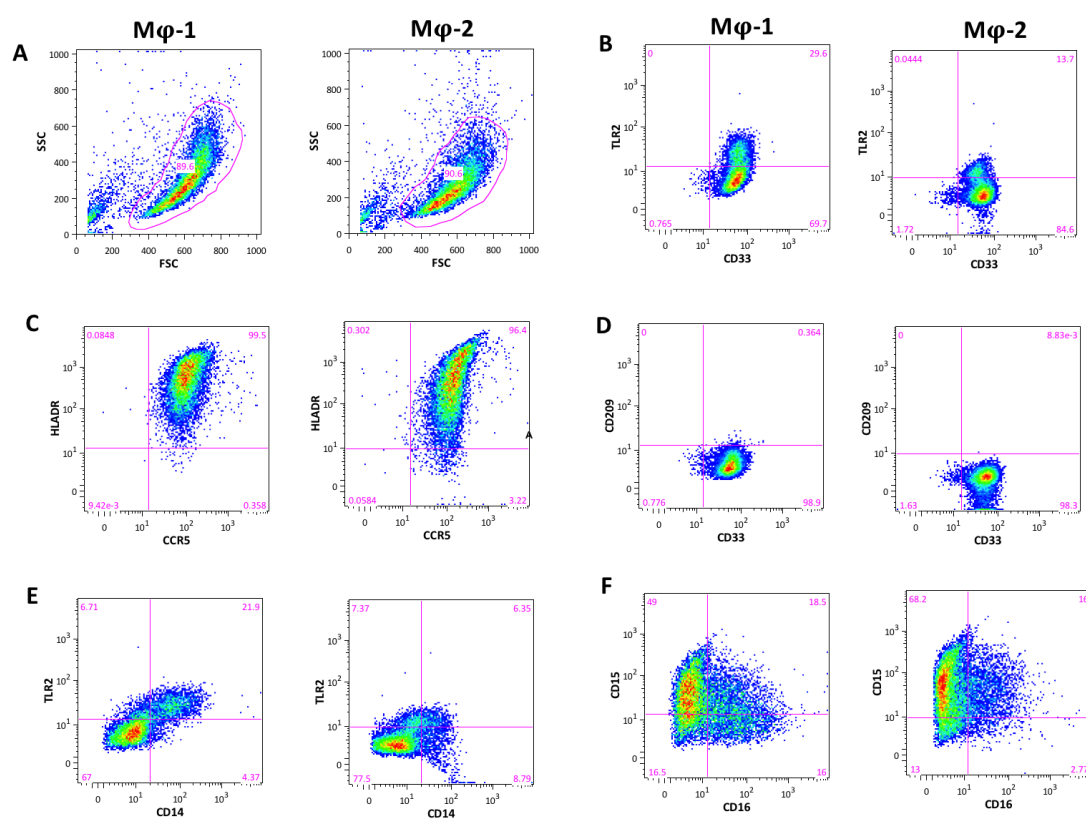


Figure 5.3 Flow cytometry dot plots of M ϕ 1 and M ϕ 2 from a representative donor. Cells were stained with labelled monoclonal antibodies (A) FSC vs. SSC (B) CD33 vs. TLR2 (C) CCR5 vs. HLADR (D) CD33 vs. CD209 (e) CD14 vs. TLR2 (F) CD16 vs. CD15

Table 5.1 Quantitative comparison of the M ϕ -1 and M ϕ -2 (n=6)

Cell surface molecule	M ϕ -1 %(SD)	M ϕ -2 %(SD)	95% CI of difference.
CD11b	99.33 (0.5)	99.25 (0.94)	-16.43 to 16.27
CD33	95.54 (7.4)	95.00 (4.2)	-18.45 to 17.37
DC-SIGN	0.41 (0.2)	0.033 (0.05)	-16.73 to 15.97
CD15	62.58 (20.1)	73.83 (22.49)	-8.777 to 31.28
CD1a	0.41 (0.1)	0.28(0.24)	-23.26 to 22.99
HLA-DR	98.62 (2.0)	92.82 (4.52)	-22.15 to 10.55
CCR5	98.23 (2.2)	96.88 (3.82)	-17.70 to 15.00
CD4	49.60 (20.6)	27.60 (24.0)	-38.35 to -5.652
TLR2	25.73 (19.5)	10.65 (6.92)	-31.43 to 1.277
CD14	55.30 (32.6)	27.88 (19.82)	-43.77 to -11.07
CD16	38.90 (12.6)	17.47 (11.03)	-37.78 to -5.082

5.2.2.2 Expression of T cell co-stimulatory and antigen presenting molecules on MDMs.

M ϕ -1 and M ϕ -2 had similar number of cells expressing HLA-DR and CD1a, on assessment by flow cytometry. Both M ϕ -1 and M ϕ -2 had large number of cells expressing of HLA-DR 98.6% and 92.8% respectively and a negligible number of cells expressing CD1a as shown in Table 5.1 and in Figure 5.4.

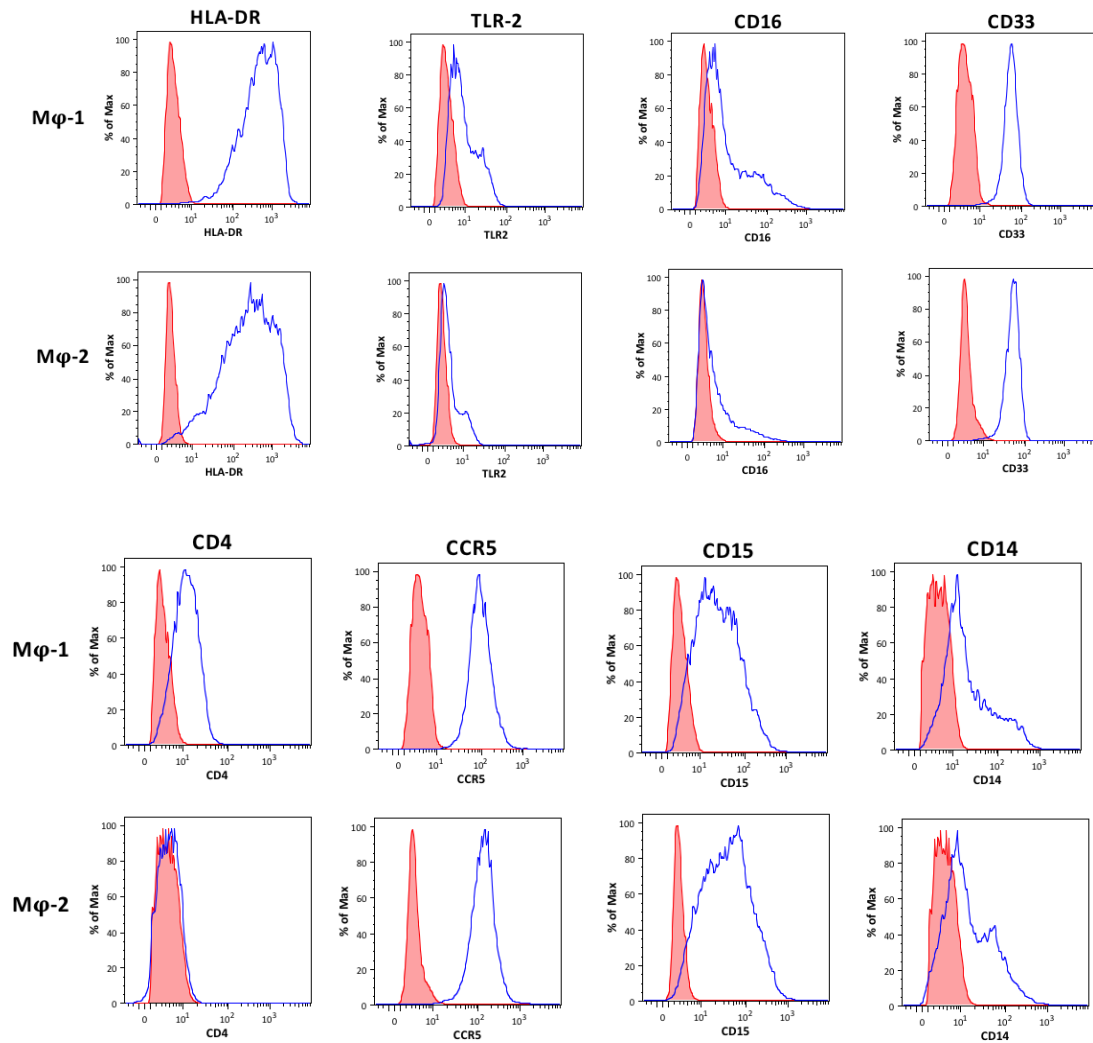


Figure 5.4 Expression of cell surface receptors on M ϕ 1 and M ϕ 2. The unshaded histograms show cells stained with specific antibody and the shaded histograms show negative controls.

5.2.2.3 Expression of adhesion molecules on MDM

Both M ϕ 1 and M ϕ 2 have high expression of CD33 and CD11b and neither of them expressed DC-SIGN Table 5.1 and Figure 5.5. Expression of CD15 was increased in M ϕ 2 than in M ϕ 1 though it was not significant at 5% level ($p > 0.05$).

5.2.2.4 Expression of pathogen pattern recognition receptors and innate immunity receptors on monocyte derived macrophages.

M ϕ -1 had higher percentage of cells expressing TLR2 (25.7% \pm 19.5 vs. 10.65 \pm 6.9), CD14 (55.3 \pm 32.6 vs. 27.9 \pm 19.8) and CD16 (38.9 \pm 12.6 vs.17.5 \pm 11) than M ϕ -2 respectively as shown in Figure 5.4 and Table 5.1. M ϕ -1 had significantly increased expression of CD14 and CD16 than in M ϕ -2 (p <0.0001 and p <0.01 respectively Figure 5.5).

5.2.2.5 Expression of HIV entry receptors CD4 and CCR5 on MDM

There was similarly increased number of cells expressing CCR5 in both M ϕ -1 and M ϕ -2 populations (98.2% \pm 2.2 and 96.9 % \pm 3.8 respectively Table 5.1). M ϕ -1 had higher expression of CD4 receptor than M ϕ -2 (49.6% \pm 20.6 and 27.6% \pm 24 respectively p <0.01 Table 5.1 and Figure 5.5).

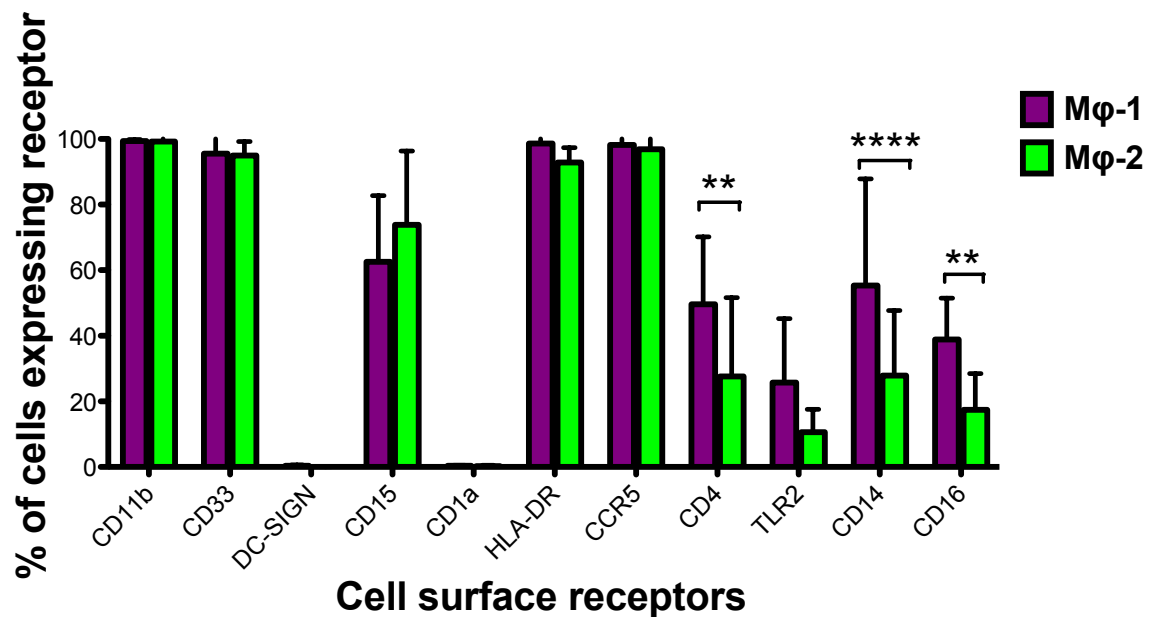


Figure 5.5 Quantitative comparison of phenotypic characteristics of M ϕ -1 and M ϕ -2 $n=6$. (Error bars mean \pm SD)

5.3 Discussion

In the present experiments, the phenotype of monocyte-derived macrophages (MDM) was determined by flow cytometry. Growth factors GM-CSF and M-CSF were used for the differentiation of monocytes into macrophages resulting in generation of M ϕ 1 and M ϕ 2 respectively. The same flow cytometer and instrument settings were used for all the analysis to allow semi-quantitative comparisons of surface receptors between donors. Effects of GM-CSF and M-CSF were compared directly in MDM from the same donor in order to eliminate donor variation. The M ϕ 1 and M ϕ 2 expressed the typical macrophage markers Mac-1 receptor (CD11b) and sialic acid binding Ig-like lectins-3 (CD33) and M ϕ 1 had significantly increased expression of CD4, CD14 and CD16 receptors compared to M ϕ 2.

Review of literature shows that variable concentrations of GM-CSF and M-CSF are used for the generation of macrophages, which results in differences in the surface receptor expression between studies. Our experiments were optimized to use 5 ng/ml GM-CSF and 20 ng/ml M-CSF. The M ϕ 1 were homogeneously round with eccentrically placed nucleus (fried egg) morphology, which is in line with results from other studies (Hassan *et al.*, 1994, Verreck *et al.*, 2006). The M ϕ 2 were round with very few numbers of spindle /fibroblastic cells which deviated from heterogeneous and fibroblastic cells reported in other studies (Bender *et al.*, 2004, Young *et al.*, 1990, Matsuda *et al.*, 1995). The high concentrations of M-CSF above 20 ng/ml used in other studies account for the heterogeneous and fibroblastic morphology of the cells. M-CSF concentration of 20 ng/ml was used in the current experiments as it is physiologically relevant and within the steady state concentration exposed to monocytes and macrophages *in vivo* (Irvine *et al.*, 2008).

Both M ϕ 1 and M ϕ 2 had similarly high expression of the adhesion receptors CD11b, CD33, and CD15, which has been reported by others (Akagawa, 2002, Finnin *et al.*, 1999, Verreck *et al.*, 2006, Xu *et al.*, 2007). The cells showed the typical absence of DC-SIGN an adhesion molecule expressed by dendritic cells (DCs).

In line with other studies both M ϕ 1 and M ϕ 2 had increased expression of HLA-DR, which regulates antigen-presenting functions to T cells (Verreck *et al.*, 2006). In previous studies GM-CSF has been reported to increase the HLA-DR expression (Dimri *et al.*, 1994, Eischen *et al.*, 1991) while in contrast M-CSF has been found to either increase or have no effect on HLA-DR expression (Becker *et al.*, 1987, Erickson-Miller *et al.*, 1990). The absence of CD1a and CD83 co-stimulatory and mediators of antigen presentation makes macrophages weak in co-stimulation and antigen presentation compared to DCs. CD1a and CD83 are typical dendritic cell markers hence their absence confirms that macrophages were generated and not DCs (Tkachenko *et al.*, 2005, Verreck *et al.*, 2006).

The increased expression of CD14 and CD16 receptors by M ϕ 1 supports their role as the main cells involved in antimicrobial response against invading pathogens. These receptor molecules are potent mediators of the innate immune response against microbial pathogens. Indeed other researchers have shown that GM-CSF generated macrophages express high levels of CD14 and CD16 (Verreck *et al.*, 2004, Fleetwood *et al.*, 2007, Becker *et al.*, 1987, Erickson-Miller *et al.*, 1990, Finnin *et al.*, 1999, Brochériou *et al.*, 2011). The levels of expression of TLR2 were also higher in M ϕ 1 though non-significantly. This is because the generated macrophages were not stimulated, TLR2 is down regulated in unstimulated macrophages and upregulated on stimulation with microbial molecules (Henning *et al.*, 2008).

The cells that were generated from monocytes with GM-CSF or M-CSF were macrophages as they expressed the typical macrophage markers: CD11b, CD33, CD14 and HLA-DR on their cell surfaces. The cells did not express any of the dendritic cells markers like CD1a, CD209, and CD83. However, the notable exception was expression of CD14, CD16 and CD4, which were significantly increased on M ϕ 1 compared to M ϕ 2. Differences in cytokine expression and phenotype of M ϕ 1 and M ϕ 2 have been described elsewhere (Verreck *et al.*, 2004), In line with this the M ϕ 1 expressed cell surface molecules associated with M1 pro-inflammatory macrophage phenotype due to the high expression of the innate antimicrobial receptors while M ϕ 2 macrophages had a lower expression of these receptors.

The overall analyses of the difference in receptor expression of the two-macrophage populations show that they can be distinguished by their cell surface receptor expression.

Chapter 6: Macrophage Phenotype Dictates Cellular Ability to Control *M. tuberculosis* And HIV-1 Replication In Response To Vitamin D

6.1 Literature review

6.1.1 Vitamin D and monocyte/macrophage biology

Macrophages are responsive to immunomodulatory molecules and their function is dependent on their phenotype, which is influenced by their microenvironment.

1,25(OH)₂D₃ modulates the immunological activities of cells of the mononuclear phagocyte system by enhancing their antimicrobial responses (Martineau *et al.*, 2008). Macrophages and monocytes are able to synthesize 1,25(OH)₂D₃ from 25-dihydroxycholecalciferol (25(OH)D₃) through expression of the CYP27B1 enzyme. The 1,25(OH)₂D₃ produced, down regulates the expression of co-stimulatory molecules CD80, CD86, CD40 and that of CD4, MHC-II, toll like receptor-2 (TLR-2), TLR4 and TLR9 on monocytes (Clavreul *et al.*, 1998, Dickie *et al.*, 2010, Xu *et al.*, 1993). On the other hand, it increases the expression of CD14 and CXCR4 (Biswas *et al.*, 1998) on monocytes.

In the presence of microbial molecules such as LPS, 25(OH)₂D₃ and 1,25(OH)₂D₃ have been shown to suppress the secretion of IL-6 and TNF-α through the inhibition of p38 activation by mitogen activated protein kinase phosphatase 1 (Zhang *et al.*, 2012). In addition, 1,25(OH)₂D₃ also upregulates the expression of macrophage specific antigens and lysosomal enzyme acid phosphatase and it enhances oxidative functions, chemotaxis and immunoglobulin- and complement-mediated phagocytic function of monocytes and macrophages (Stoffels *et al.*, 2006, Abu-Amer and Bar-Shavit, 1993, Xu *et al.*, 1993). IL-4, on the other hand, antagonises the effects of 1,25(OH)₂D₃ on monocytes and macrophages (Xu *et al.*, 1993).

Activation of macrophages by interferon gamma (IFN-γ) or through TLR induction with microbial molecules increases the expression of CYP27B1.

In turn this is involved in synthesis of $1,25(\text{OH})_2\text{D}_3$ from $25(\text{OH})\text{D}_3$, which induces the synthesis of the antimicrobial peptides, cathelicidin and β defensins, that restrict the growth of intracellular pathogens such as *Mycobacterium tuberculosis* (Martineau *et al.*, 2007b, Fabri *et al.*, 2011, Wang *et al.*, 2004).

$1,25(\text{OH})_2\text{D}_3$ synthesis in macrophages is not tightly regulated as it is dependent on the availability of the substrate $25(\text{OH})\text{D}_3$. $1,25(\text{OH})_2\text{D}_3$ induces the expression of the truncated splice variant of CYP24 by macrophages that is not able to degrade $1,25(\text{OH})_2\text{D}_3$. It only binds it, preventing its degradation, and thereby increasing availability of $1,25(\text{OH})_2\text{D}_3$ for cell functions (Ren *et al.*, 2005). This is in contrast to the renal production of $1,25(\text{OH})_2\text{D}_3$, which is controlled by negative feedback on CYP27B1 (Adams and Gacad, 1985).

6.1.2 Vitamin D signalling and HIV-1 long terminal repeat (LTR)

The HIV-1 LTR is a sequence of nucleotides in the HIV-1 genome that have binding sites for molecules that control HIV-1 replication. It is responsive to different types of molecules; some of which stimulate and others inhibit HIV-1 replication. *In vivo* and *in vitro* HIV-1 and *M. tuberculosis* co-infection has been shown to stimulate the secretion of molecules that stimulate signalling pathways that result in modulation of the HIV-1 LTR.

The HIV-1 long terminal repeat (LTR) has three parts: U3, R and U5 as shown in Figure 6.1. The U3 area has the promoter, enhancer and regulatory sequences, while the R region contains the transactivator response element (TAR) (Stevens *et al.*, 2006). The LTR sequences interact with host transcriptional factors, chromatin remodelling factors and viral proteins to induce or inhibit expression of viral RNA and proteins.

The processes that are under LTR control include viral replication, expression of viral proteins, assembly and transition from latency to productive infection.

HIV-1 has two LTR; 5'-LTR and 3'-LTR, which have identical sequences and structural organisation but different functions. The 5'-LTR is involved in induction of HIV-1 transcription while the 3'-LTR is important for termination of transcription, cleavage and polyadenylation of viral mRNA (Klaver and Berkhout, 1994).

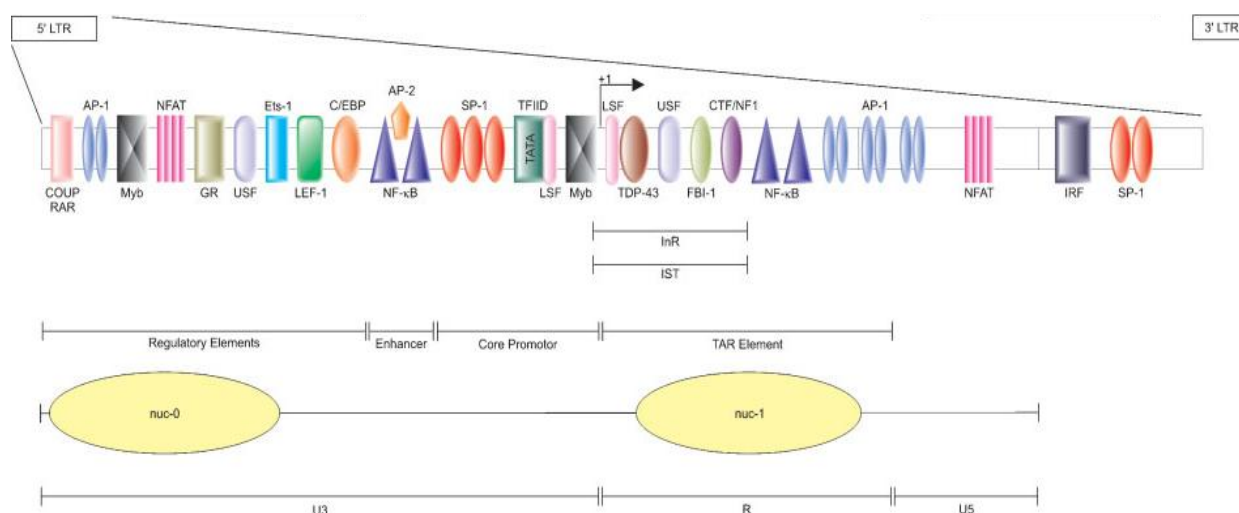


Figure 6.1 Structure of the HIV-1 5'-LTR of HIV-1 subtype B (Stevens *et al.*, 2006)

Interaction of host transcriptional factors and viral proteins with the HIV-1 5'-LTR stimulates transcription of viral DNA by RNA polymerase II (pol II). The LTR contains binding sites for specific proteins like; nuclear factor kappa beta (NF-κβ), CCAAT/enhancer binding proteins (C/EBP), nuclear factor of activated T cells (NFAT), activator protein (AP-1) and activating transcriptional factor (ATF) in the U3 and U5 regions. The number and arrangement of these binding sites varies between HIV-1 subtypes (Stevens *et al.*, 2006).

Initiation of viral transcription is induced in the core promoter that contains three specific protein-1 (sp-1) binding sites, TATA box and initiator element (Kilareski *et al.*, 2009). The initiator element interacts with a number of host molecules that can either stimulate or inhibit basal HIV-1 transcription. In the enhancer region, there are two binding sites for proteins of the Rel family and NF-κβ. Binding of the NF-κβ to the HIV-1 enhancer region increases HIV-1 replication and expression of HIV-1 proteins in T lymphocytes and macrophages (Rabson and Lin, 2000).

HIV-1 subtype C has three, subtype B has two and subtype E has one NF- κ B binding sites. These are the principal binding sites of NF- κ B, whose p65 subunit is a strong inducer of HIV-1 transcription (Natoli *et al.*, 2005). More NF- κ B binding sites in subtype C results in its higher level of basal and TNF- α induced transcriptional activity (Montano *et al.*, 2000).

NF- κ B binding sites also bind nuclear factor of activated T cells (NFAT), resulting in stimulation of transcription in T lymphocytes. NFAT-5 is important in induction of basal transcription and HIV-1 replication in monocytes and monocyte derived macrophages (Bates *et al.*, 2008). The regulatory and TAR also contains the binding sites for transcriptional factors that target the mitogen activated protein kinase (MAPK) signal transduction pathways such as activating protein (AP-1).

1,25(OH)₂D₃ has been shown to down regulate NF- κ B mediated signalling by inhibiting its translocation to the nucleus in a number of cells types, including monocytes and macrophages (Cohen-Lahav *et al.*, 2006, Dong *et al.*, 2003). Consequently, this results in the suppression of the expression of gene products that are under its control. NF- κ B is composed of two subunits; p50 and p65, which under normal conditions stay in the cytoplasm bound to inhibitory kappa beta (I κ B). Activation of cells results in phosphorylation and break down of I κ B resulting in release of NF- κ B, which migrates to the nucleus where it modulates target genes. 1,25(OH)₂D₃ modulates NF- κ B functions by increasing the levels of I κ B and by inhibition of I κ B phosphorylation. It also prevents the binding of NF- κ B to the promoters of its target genes (D'Ambrosio *et al.*, 1998, Harant *et al.*, 1998, Cohen-Lahav *et al.*, 2006).

6.1.3 Effects of vitamin D on HIV-1 and *M. tuberculosis*

There are conflicting reports on the effects of vitamin D molecules 25(OH)D₃ and 1,25(OH)₂D₃ on HIV-1 replication. Previous studies have mainly been carried out in myeloid cells lines, while very few have used primary macrophages.

Studies that report increased HIV-1 replication by 1,25(OH)₂D₃ were primarily performed in cell lines (including HL-60, U937, THP-1, A3.5 and U1 cells) after pre-treatment of the cells prior to HIV-1 infection. HIV-1 replication in these experiments was determined by the measurement of p24 levels (Kitano *et al.*, 1990, Pauza *et al.*, 1993, Skolnik *et al.*, 1991) and HIV-1 proviral DNA reverse transcriptase activity (Biswas *et al.*, 1998). However, addition of 1,25(OH)₂D₃ to cells post-infection with HIV-1, results in inhibition of HIV-1 replication (Pauza *et al.*, 1993). In cells with constitutively low viral expression due to integration of proviral DNA into their genome (chronically HIV-1 infected cells such as U937 and J-HL-60), 1,25(OH)₂D₃ increases HIV-1 replication (Locardi *et al.*, 1990, Kitano *et al.*, 1990). In contrast, 1,25(OH)₂D₃ suppressed HIV-1 replication in chronically HIV-1 infected U1 cells and the addition of exogenous TNF-α increased HIV-1 replication (Goletti *et al.*, 1995).

The effect of 1,25(OH)₂D₃ on primary monocyte derived macrophages (MDM) has also produced variable results. Pre-treatment of MDM with 1,25(OH)₂D₃ has been shown to increase HIV-1 replication (Skolnik *et al.*, 1991, Kizaki *et al.*, 1993) and the addition of 1,25(OH)₂D₃ post-infection with HIV-1, results in inhibition of HIV-1 replication (Kizaki *et al.*, 1993). The effect of 1,25(OH)₂D₃ on HIV-1 in MDM was found to vary between donors, as pre-treatment with 1,25(OH)₂D₃ resulted in inhibition of HIV-1 replication in most of the donors, but in a few, it resulted in increased HIV-1 replication which was, however, less than that seen in myeloid cell lines (Pauza *et al.*, 1993). In addition, the binding of 1,25(OH)₂D₃ to VDR in HeLa, Cos-1 and U937 cell lines has been shown to transactivate HIV-1 LTR in combination with its co-activators, which stimulate HIV-1 replication (Nevado *et al.*, 2007). However, more recently, Campbell and Spector demonstrated that 1,25(OH)₂D₃ is able to inhibit both HIV-1 and *M. tuberculosis* replication by autophagy even during co-infection (Campbell and Spector, 2012).

A number of *in vitro* studies have shown that 1,25(OH)₂D₃ and 25(OH)D₃ are able to restrict the growth of *M. tuberculosis* in monocytes and macrophages.

Several mechanisms are used to inhibit growth of *M. tuberculosis* in the presence of vitamin D, for instance, the induction of the synthesis of antimicrobial peptides cathelicidin and β defensins in phagocytes that lyse the bacilli by the formation of pores in their cell wall and membranes (Martineau *et al.*, 2007b, Liu *et al.*, 2006). Other mechanisms include the induction of autophagy, increased synthesis of ROS, RNI, and inhibition of secretion of MMP that are involved in tissue destruction (Sly *et al.*, 2001, Yuk *et al.*, 2009, Coussens *et al.*, 2009). Further, vitamin D has been shown to inhibit coronin, thereby enhancing the maturation and fusion of the phagosome containing *M. tuberculosis* together with the lysosomes and results in its degradation in macrophages (Anand and Kaul, 2003).

In cognisance of the inconclusive results of the effect of vitamin D on HIV-1 replication and in order to examine the safety of vitamin D supplementation in HIV-1 infected individuals in primary monocyte derived macrophages, I next investigated the effect of pre-treatment of macrophages with 25(OH)D₃ and 1,25(OH)₂D₃ on HIV-1 replication. Additional factors, including macrophage phenotype and co-infection with *M. tuberculosis*, were also investigated.

6.2 Results

6.2.1 The effect of vitamin D on HIV-1 replication

To determine the effect of vitamin D pre-treatment on HIV-1 replication in phenotypically distinct macrophages, supernatant p24 concentrations were measured 144 hrs and 240 hrs after HIV-1 infection of both M ϕ 1 and M ϕ 2.

Two-way repeated measure ANOVA was used for analysis of the results. There was a significant effect of 25(OH)D₃ and 1,25(OH)₂D₃ after 144hrs which, inhibited HIV-1 replication in M ϕ 1 ($p=0.01$). Macrophage phenotype had no effect on HIV-1 replication ($p=0.73$) nor was there an interaction between macrophage phenotype and vitamin D molecules on HIV-1 replication ($p=0.43$).

In order to determine where the effect of vitamin D molecules was located, additional post-hoc pairwise ANOVA comparisons with Bonferroni correction of multiple comparison were used within each macrophage phenotype. Vitamin D significantly inhibited HIV-1 growth in M ϕ -1 ($p=0.0009$), with a greater significance of the effect seen with 1,25(OH) $_2$ D3 than 25(OH)D3 ($p<0.001$ and $p<0.05$, respectively) pre-treatment. Conversely, there was no significant effect of either vitamin D molecules on the growth of HIV-1 in the M ϕ -2 ($p=0.21$) after 144 hrs (Figure 6.2A).

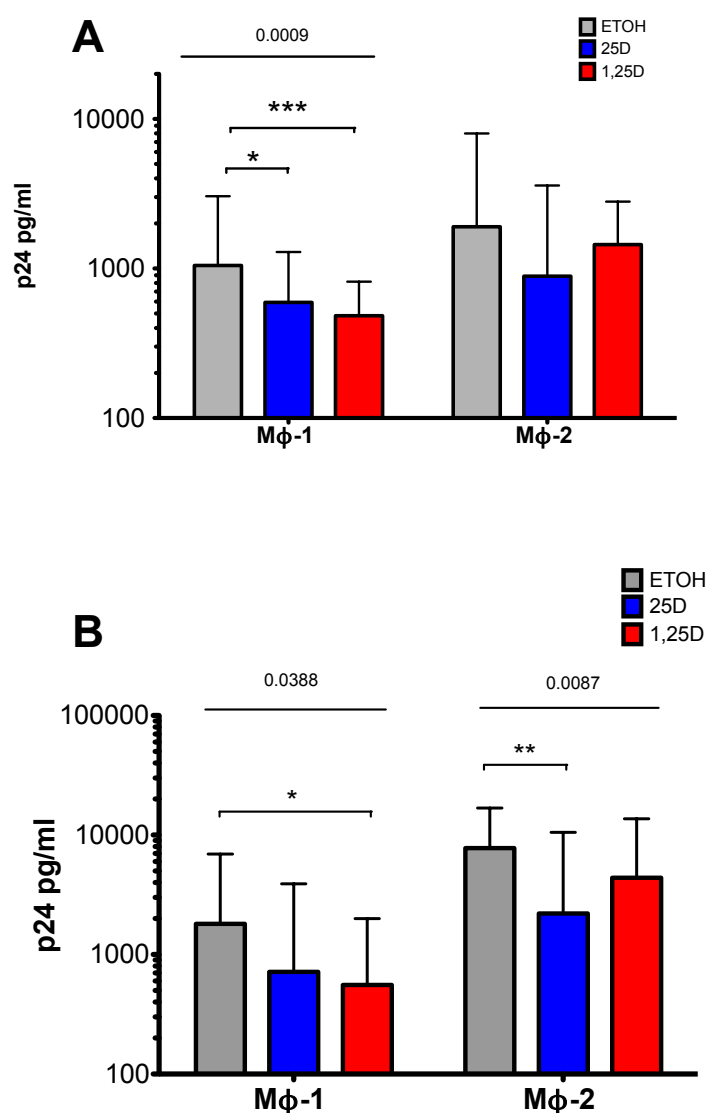


Figure 6.2. The effect of 1,25(OH) $_2$ D3 on HIV-1 replication after (A) 144 hrs and (B) 240 hrs of infection. Bars represent median and error bars IQR ($n=12$); repeated measures 1way ANOVA with Bonferroni post-hoc correction, * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

After 240 hrs there was a significant difference in inhibition of HIV replication after treatment with vitamin D molecules in M ϕ -1 ($p = 0.0388$) and 1,25(OH) $_2$ D $_3$ significantly restricted HIV replication ($p < 0.05$). In M ϕ -2 there was significant inhibition of HIV replication in the presence of vitamin D molecules ($p = 0.0087$) and 25(OH)D $_3$ significantly restricted HIV replication ($p < 0.01$) (Figure 6.2B).

6.2.2 The effect of vitamin D on HIV-1 replication during co-infection with *M. tuberculosis*.

As previous studies have shown, co-infection with *M. tuberculosis* enhances HIV-1 replication. The effect of vitamin D molecules on HIV-1 replication in macrophages co-infected with *M. tuberculosis* was therefore assessed. The effect of vitamin D molecules on HIV-1 replication was evaluated by two way repeated measure ANOVA after 72 hrs of HIV-1 and *M. tuberculosis* co-infection of macrophages. The vitamin D molecules restricted HIV-1 replication in M ϕ -1 ($p=0.01$). On the other hand, co-infection with *M. tuberculosis* had no effect on HIV-1 replication ($p=0.54$). There was no interaction of vitamin D molecules and co-infection with *M. tuberculosis*, to influence HIV-1 replication ($p=0.51$). Further pair-wise multiple comparisons revealed a significant difference in the growth of HIV-1 in M ϕ -1 macrophages co-infected with *M. tuberculosis* ($p=0.006$). Both 25(OH)D $_3$ and 1,25(OH) $_2$ D $_3$, significantly restricted HIV-1 replication during co-infection with *M. tuberculosis* ($p < 0.05$ in both cases) (Figure 6.3A).

In the M ϕ -2, HIV-1 replication was not influenced by vitamin D molecules, nor by co-infection with *M. tuberculosis* ($p=0.16$ and $p=0.58$ respectively). There was also no interaction between these two independent variables to influence HIV-1 replication ($p=0.38$). One-way ANOVA showed that there was no significant difference in the HIV-1 p24 levels for the macrophages co-infected with *M. tuberculosis* in the presence of vitamin D molecules ($p=0.21$) (Figure 6.3B).

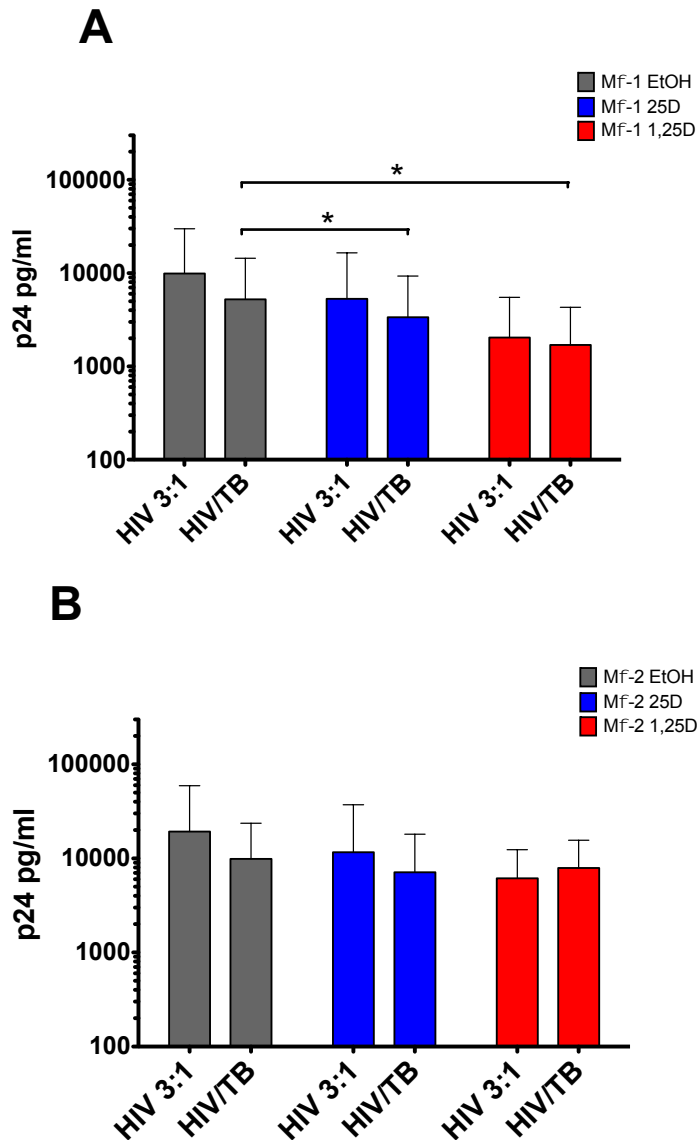


Figure 6.3. Effect of 25(OH)D3 and 1,25(OH)₂D3 on HIV-1 replication 72 hrs after coinfection with or without *M. tuberculosis*. (A) Mφ1 and (B) Mφ2 Median is plotted with IQR (n=12). One way ANOVA with Bonferroni post-hoc correction, * = p<0.05, ** = p<0.001.

6.2.3 The effect of vitamin D on Uptake of *M. tuberculosis*

1,25(OH)₂D3 has previously been shown to enhance phagocytosis by macrophages. I therefore studied the effect of vitamin D molecules on uptake of *M. tuberculosis* after 4 hrs of infection and used two way repeated measure ANOVA for analysis of the results. 1,25(OH)₂D3 and 25(OH)D3, did not have an effect on the phagocytosis of *M. tuberculosis* by Mφ1 (p=0.47) and Mφ2 (p=0.61).

Also, the macrophage phenotype had no effect on the uptake of *M. tuberculosis* in the presence of vitamin D in HIV-1-infected and HIV-1-uninfected macrophages ($p=0.99$ and $p=0.81$ respectively). In addition, co-infection with HIV-1 did not influence the phagocytosis of *M. tuberculosis* by M ϕ 1 and M ϕ 2 ($p=0.92$ and 0.61 respectively, Figure 6.4A and B).

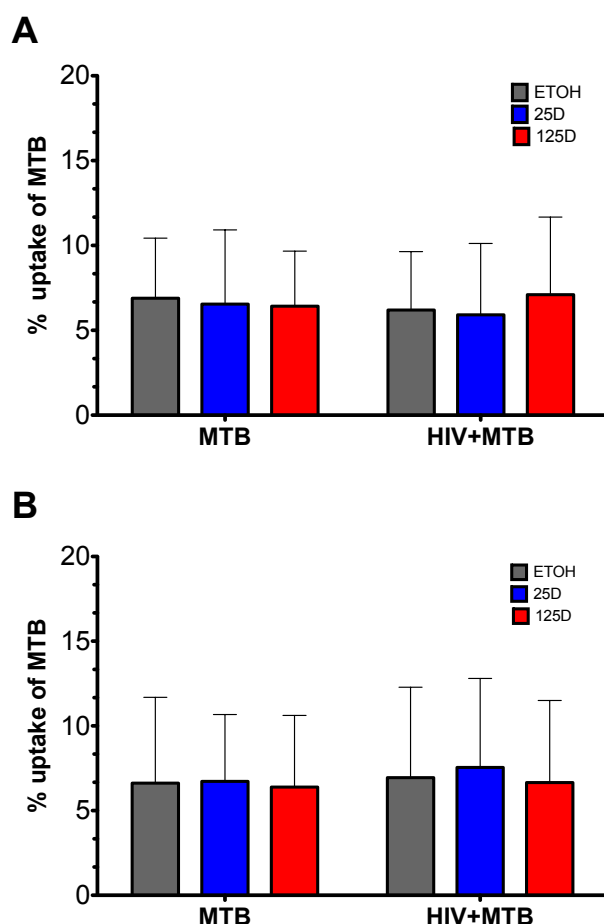


Figure 6.4. The effect of vitamin D molecules on the uptake of *M. tuberculosis* in (A) M ϕ 1 and (B) M ϕ 2 grown for 168 hrs in either the presence or absence of HIV-1 infection . Mean is plotted with SD ($n=6$).

6.2.4 The effect of vitamin D on the growth of *M. tuberculosis*.

The growth of *M. tuberculosis* in the presence of vitamin D and HIV-1 co-infection was monitored over 168 hrs in M ϕ 1 and M ϕ 2 (Figure 6.5A and B).

M. tuberculosis growth was significantly suppressed in HIV-1 infected M ϕ 1 after 168 hrs in the presence of 25(OH)D3 and 1,25(OH)₂D3 compared to the controls ($p < 0.05$ in both cases). In addition 1,25(OH)₂D3 also restricted the growth of *M. tuberculosis* in HIV-1 uninfected M ϕ 1 ($p < 0.05$).

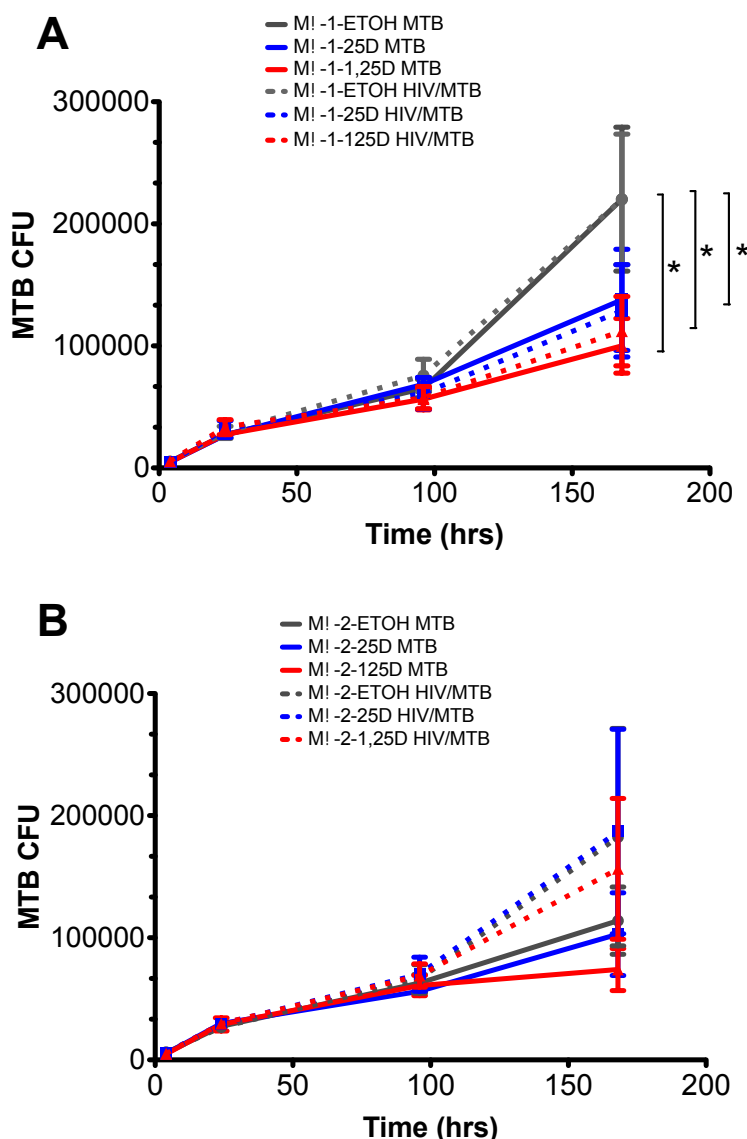


Figure 6.5. Growth of *M. tuberculosis* over 168 hrs in the presence or absence of vitamin D and or HIV-1 in (A) M ϕ 1 and (B) M ϕ 2. Bars represent mean \pm SD ($n=6$). * = $p < 0.05$, ** = $P < 0.01$, *** = $p < 0.001$.

At the 24 hrs and 96 hrs time points neither vitamin D molecules nor co-infection with HIV-1 had significant effect on the growth of *M. tuberculosis* in both M ϕ 1 and M ϕ 2 ($p > 0.05$ in all cases, Figure 6.6).

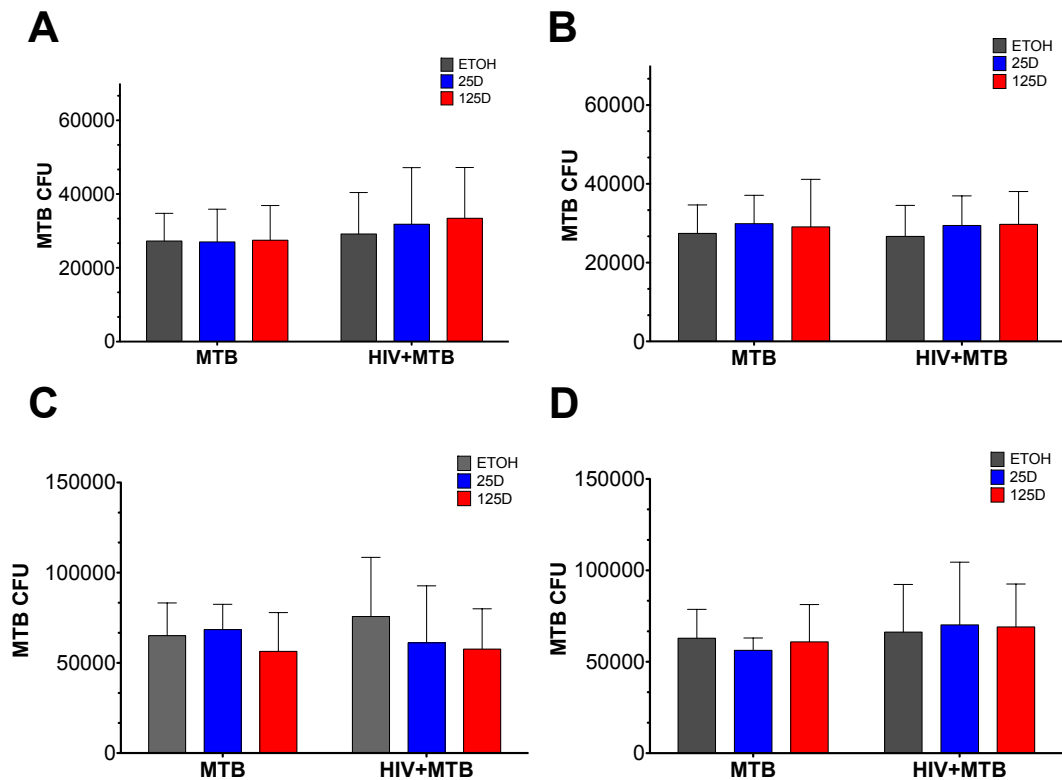


Figure 6.6. The effect of vitamin D on intracellular *M. tuberculosis* (MTB) growth at 24 hrs (A-B) and 96 hrs (C-D) post- *M. tuberculosis* infection of Mφ1 (A,C) and Mφ2 (B,D) in the presence (HIV+MTB) or absence (MTB) of HIV-1 infection 168 hrs pre- *M. tuberculosis* infection. Bars represent mean ± SD (n=6).

Two-way repeated measure ANOVA was used to determine the effect of vitamin D molecules and co-infection with HIV-1 on the growth of *M. tuberculosis* at the 168 hrs time point in Mφ1. The analysis revealed a significant effect of vitamin D on the growth of *M. tuberculosis* ($p=0.001$) during mono-infection. Co-infection with HIV had no effect on *M. tuberculosis* growth ($p=0.99$) and there was no interaction between vitamin D molecules and co-infection on the growth of *M. tuberculosis* ($p=0.93$, Figure 6.7A).

Post hoc analysis showed that there was a significant difference in the growth of *M. tuberculosis* in HIV-1 uninfected Mφ1 ($p=0.0521$) and HIV-1 infected Mφ1 ($p=0.0081$) in the presence of vitamin D molecules (Figure 6.7A).

In the HIV-1 uninfected M ϕ 1 only, 1,25(OH)₂D₃ significantly inhibited the growth of *M. tuberculosis* ($p < 0.05$). Whereas in HIV-1-infected M ϕ 1, both 25(OH)D₃ and 1,25(OH)₂D₃ significantly restricted the growth of *M. tuberculosis* ($p < 0.05$ in both cases). Vitamin D molecules or co-infection with HIV-1 had no significant effect on the growth of *M. tuberculosis* in the M ϕ 2, ($p = 0.25$ and $p = 0.79$ respectively, Figure 6.7B).

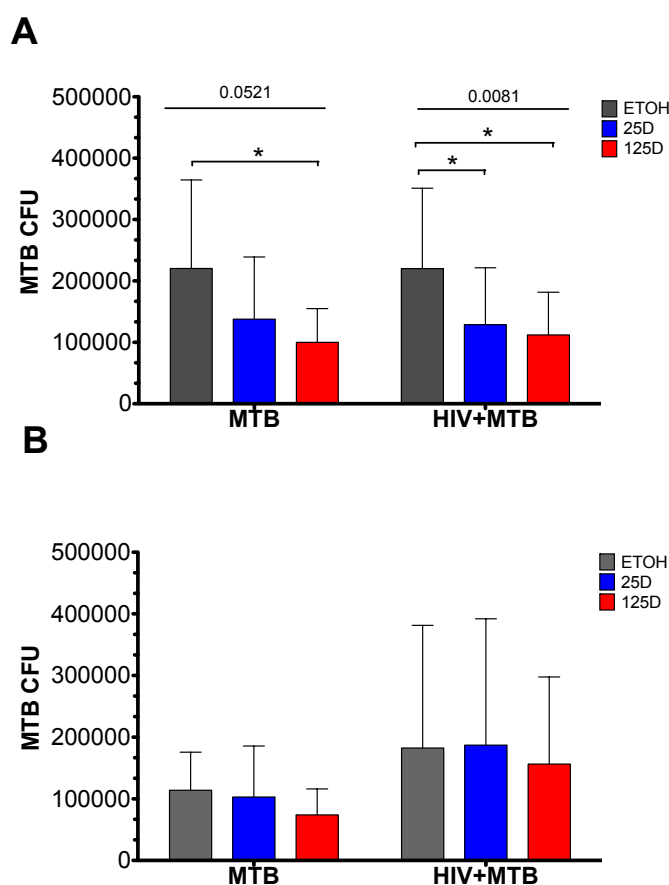


Figure 6.7. The effect of vitamin D on intracellular *M. tuberculosis* (MTB) after 168 hrs in (A) M ϕ 1 and (B) M ϕ 2 in the presence or absence of HIV-1 co-infection (HIV+MTB). Error bars represent mean \pm SD ($n=6$). (* = $p < 0.05$).

6.2.5 The effect of macrophage phenotype on *M. tuberculosis* growth

The effect of macrophage phenotype and vitamin D molecules on the growth of *M. tuberculosis* was determined after 168 hrs.

There was no effect of macrophage phenotype on *M. tuberculosis* growth in HIV-1 infected ($p=0.82$) and uninfected ($p=0.41$) macrophages. Finally, there was no interaction between macrophage phenotype and vitamin D molecules in HIV-1 infected ($p=0.27$) and uninfected ($p=0.55$) macrophages.

6.3 Discussion

The safety of vitamin D supplementation has been a question of interest as inconclusive results on the effect of vitamin D on HIV-1 replication have been reported by various studies. Here, I investigated the effect of vitamin D supplementation during HIV-1, MTB and HIV-1/MTB co-infection using pro-inflammatory ($M\phi 1$) and anti-inflammatory ($M\phi 2$) macrophages generated from primary human monocytes.

I found that vitamin D molecules, 25(OH)D3 and 1,25(OH)₂D3, were effective in suppressing HIV-1 replication in pro-inflammatory macrophages infected with HIV-1 only and during co-infection with *M. tuberculosis*. In addition, 25(OH)D3 and 1,25(OH)₂D3, significantly restricted the growth of *M. tuberculosis* in HIV-1 infected and HIV-1 uninfected pro-inflammatory macrophages. 25(OH)D3 only inhibited HIV replication while both 25(OH)D3 and 1,25(OH)₂D3, had no effect on HIV-1 replication and *M. tuberculosis* growth during co-infection in $M\phi 2$.

For the past fifty years there have been no new tuberculosis drugs on the market, although a number of drugs are currently in development. In recent years, the prevalence of multidrug resistant and extensively drug resistance tuberculosis has been on the rise, due to a number of factors; including social and economic factors, high prevalence of HIV-1, non-adherence to treatment and inaccessibility to consistent medical care (Cox *et al.*, 2010). This has necessitated the need to explore the use of adjunct immunotherapy to enhance the host immune response against *M. tuberculosis*.

Vitamin D is one of the immunotherapeutic agents that has been shown to enhance the host innate immune response against *M. tuberculosis* (Martineau *et al.*, 2007a). Vitamin D also has anti-inflammatory functions which help resolve pathology better than normal treatment alone (Coussens *et al.*, 2012).

The finding that pre-treatment of MDM with vitamin D molecules 25(OH)D₃ and 1,25(OH)₂D₃ results in restriction of HIV-1 is contrary to other studies which showed that pre-treatment stimulates HIV-1 replication (Skolnik *et al.*, 1991, Kizaki *et al.*, 1993, Kitano *et al.*, 1990). In line with what was reported by Pauza *et al.*, in monocyte-derived macrophages, there was a variation of the effect of the vitamin D molecules among the donors. Most of the donors inhibited HIV-1 replication, but a few of them failed to inhibit HIV-1 replication in the anti-inflammatory Mφ2 (Pauza *et al.*, 1993). The ability of 1,25(OH)₂D₃ to suppress growth of HIV-1 has also been reported in monocyte derived macrophages during co-infection with *M. tuberculosis* (Campbell and Spector, 2012). This study also found no effect of co-infection on HIV-1 replication in response to Vitamin D. In contrast, many studies using cell lines like Cos-1, U937, U1, HeLa and A3.5 have reported increased HIV-1 replication in the presence of 1,25(OH)₂D₃ (Nevado *et al.*, 2007, Biswas *et al.*, 1998, Skolnik *et al.*, 1991, Pauza *et al.*, 1993).

The variation in results may relate to the use of different HIV-1 strains with different receptor tropism, differences in time points where the measurement of virus concentration was performed, the use of different cell lines, some of which produce cytokines that activate HIV-1 replication, the use of different concentrations of vitamin D molecules, the purity of isolated cells (if T cells are present or not), the maturation state and the period that cells are treated with vitamin D molecules pre- or post-HIV-1 infection .

The mechanisms by which vitamin D is able to inhibit HIV-1 replication is not yet fully understood. Since vitamin D has been shown to inhibit TNF- α secretion (Martineau *et al.*, 2007b), the suppression of HIV-1 replication by vitamin D might be through its inhibition of the TNF- α and NF- κ B signaling pathways that activate HIV-1 replication through binding of NF- κ B to the HIV-1 LTR (Lee *et al.*, 2005, Cohen-Lahav *et al.*, 2006). Indeed, 1,25(OH)₂D₃ has been shown to inhibit Respiratory Syncytial Virus (RSV) replication through suppression of the NF- κ B pathway, by increasing the levels of I κ B and its stability in RSV-infected cells (Hansdottir *et al.*, 2010). Moreover, 1,25(OH)₂D₃ has also been shown to inhibit HIV-1 replication by mediating enhanced autophagy, which results in the killing of HIV-1 (Campbell and Spector, 2012).

Despite the *in vitro* studies that report increased HIV-1 replication in the presence of vitamin D, there are no *in vivo* studies that support the result. A number of *in vivo* studies, where vitamin D has been provided to HIV-1-infected individuals, resulted in no adverse effects, and had no effect on viral load and CD4⁺ cell counts (Arpadi *et al.*, 2012, Arpad *et al.*, 2009, Kakalia *et al.*, 2011, Havens *et al.*, 2012). Moreover, some supplementation studies even show increased CD4⁺ cell count and better survival in HIV-1-infected individuals with higher vitamin D levels (Mehta *et al.*, 2010, Mehta *et al.*, 2011).

In the current study, 100 nM 25(OH)D₃ and 100 nM 1,25(OH)₂D₃ significantly restricted *M. tuberculosis* growth in HIV-1 infected pro-inflammatory M ϕ 1, whereas only 1,25(OH)₂D₃ treatment significantly restricted the growth of *M. tuberculosis* in HIV-1-uninfected M ϕ 1. This is in line with a number of studies that have demonstrated that vitamin D inhibits the growth of *M. tuberculosis* (Liu *et al.*, 2006, Martineau *et al.*, 2007b). Vitamin D has been demonstrated to restrict the growth of *M. tuberculosis* through a number of mechanisms which include; direct lysis by pore-forming antimicrobial peptides cathelicidin and β -defensins, autophagy, which is enhanced by cathelicidin, enhanced fusion of *M. tuberculosis* phagosomes with lysosomes, increased production of ROI and RNI and also by inhibition of the synthesis of coronin (Yuk *et al.*, 2009, Fabri *et al.*, 2011, Anand and Kaul, 2003, Liu *et al.*, 2006).

In addition, vitamin D has been shown to restrict growth of *M. tuberculosis* by increasing the production of ROS and in the mouse model has been shown to induce production of RNI (Yang *et al.*, 2009, Thoma-Uszynski *et al.*, 2001).

Furthermore TLR2/1 stimulation by *M. tuberculosis*, results in secretion of IL-15, which induces IL-32 and subsequently activates the vitamin D dependent antimicrobial pathway, which restricts *M. tuberculosis* growth (Marcos Munoz *et al.*, 2012, Krutzik *et al.*, 2008). IL-32 has previously been shown to inhibit growth of *M. tuberculosis* in macrophages (Bai *et al.*, 2010). The hypoxic conditions, such as those encountered in the granuloma during latent tuberculosis, have also been shown to activate the production of cathelicidin and β -defensins via the vitamin D antimicrobial pathway that result in suppression of *M. tuberculosis* growth (Nickel *et al.*, 2012).

The mechanism by which 25(OH)D3 and 1,25(OH)2D3 restrict the growth of both HIV-1 and *M. tuberculosis* during co-infection is not yet fully understood but the antimicrobial peptides cathelicidin and defensins as well as autophagy induced by *M. tuberculosis* infection might also inhibit HIV-1 replication during co-infection (Campbell and Spector, 2012, Yuk *et al.*, 2009). Both cathelicidin and β -defensins have been shown to inhibit HIV-1 replication (Bergmana *et al.*, 2007, Seidel *et al.*, 2010). In addition IL-32, which is also secreted in response to *M. tuberculosis* by the macrophages, has also been shown to restrict HIV-1 replication (Nold *et al.*, 2008, Marcos Munoz *et al.*, 2012). All these pathways might be working synergistically to inhibit the growth of both HIV-1 and *M. tuberculosis* in macrophages.

Vitamin D molecules did not have an effect on the phagocytosis of *M. tuberculosis* by pro-inflammatory and anti-inflammatory macrophages. A previous study by Chandra *et al.*, found that 1,25(OH)2D3 increases phagocytosis of *M. tuberculosis* by macrophages with a low phagocytic index but not for those with a high phagocytic index (Chandra *et al.*, 2004). Macrophage phenotype had no significant effect on the growth of HIV-1 or *M. tuberculosis*.

The similar growth of HIV-1 in pro-inflammatory M ϕ 1 and anti-inflammatory M ϕ 2 might be due to the anti-inflammatory effects of vitamin D on the pro-inflammatory macrophages making them more like the anti-inflammatory macrophages. The pro-inflammatory M ϕ 1 were more effective in significantly restricting the growth of HIV-1 and *M. tuberculosis* compared to the anti-inflammatory M ϕ 2 when treated with vitamin D. This has clinical relevance *in vivo*, since alveolar macrophages have a pro-inflammatory phenotype due to their exposure to high levels of GM-CSF in lung tissue as detected in the mouse model (Trapnell and Whitsett, 2002). In healthy donors, GM-CSF levels are detectable and are increased on exposure to microbes (Akagawa *et al.*, 2006, Aggarwal *et al.*, 2000, Chen *et al.*, 2001, Gomez and Prince, 2008).

Even though 25(OH)D₃ and 1,25(OH)₂D₃ down modulate pro-inflammatory cytokines like TNF- α , IL-12, IL-6 and IL-1 β which play a role in immunity to HIV-1 and *M. tuberculosis*, they are able to enhance the ability of the innate immune response to control pathogens. In the presence of the vitamin D molecules, the immune system stimulates the antimicrobial pathways that are effective in the elimination of HIV-1 and *M. tuberculosis*. Also, the modulation of the pro-inflammatory immune response prevents tissue damage by allowing clearance of the pathogens in competent tissue. *In vivo* supplementation of vitamin D has predominant anti-inflammatory effects, which benefit the host during infection (Coussens *et al.*, 2012).

Primary monocyte derived macrophages give a better reflection of the interaction of HIV-1 and *M. tuberculosis* in macrophages *in vivo* in the presence of vitamin D molecules compared to myeloid and osteoblastic cell lines. This is because cell lines do not accurately mimic the behavior of tissue macrophages and might produce different amounts and types of cytokines that might stimulate HIV-1 replication as they are derived from malignant cells. For example increased activation of NF- κ B, which also induce HIV-1 replication is found in many malignant cells where it is a major activator of cancer cell proliferation and survival as it activates many pro-survival and anti-apoptotic genes (Wang *et al.*, 2012, Rushworth *et al.*, 2010).

The limitation of the macrophage model is the absence of other cells, as macrophages in the host interact with other immune cells. There is therefore a need for further investigations in a model where T lymphocytes are included in the model in order to better mimic the situation in the lungs.

Bronchoalveolar lavage fluid from HIV infected persons have low levels of 25(OH)D₃ than those from HIV uninfected persons (Anandaiah *et al.*, 2012). Therefore increased vitamin D levels might result in effective inhibition of both HIV-1 and *M. tuberculosis* growth in lungs of HIV-1 infected individuals. *M. tuberculosis* strains such as CDC1551 have been shown to increase HIV-1 replication through enhanced production of NF- κ B through the effects of the Rv0652 protein, which stimulates the secretion of pro-inflammatory cytokines and activates NF- κ B signalling (Kim *et al.*, 2012). Vitamin D supplementation might inhibit HIV-1 replication by down regulating the expression of NF- κ B in HIV-1-infected individuals infected with this strain thereby ameliorating disease outcome. There is also a need for the evaluation of the effect of vitamin D together with tuberculosis drugs on the growth of multidrug resistant and extremely drug resistant strains of *M. tuberculosis* in HIV-1-infected and HIV-1-uninfected infection models. This is because vitamin D induces the production of pore-forming antimicrobial peptides such as cathelicidin and defensins, increased production of ROS and RNI and inhibition of coronin damages the bacilli and can possibly enhance the ability of anti-tuberculosis drugs to kill the drug resistant bacteria (Martineau *et al.*, 2007b, Sly *et al.*, 2001, Anand and Kaul, 2003, Rathored *et al.*, 2012). Further investigations to determine the effects of 25(OH)D₃ and 1,25(OH)₂D₃ on different strains of *M. tuberculosis* are also needed.

In conclusion, 25(OH)D₃ and 1,25(OH)₂D₃ inhibited HIV-1 and *M. tuberculosis* growth in M ϕ -1 and 25(OH)D₃ inhibited HIV replication in M ϕ -2, taken together with the currently available *in vivo* data, this supports the use of vitamin D by HIV-1-infected individuals for the prevention of activation of tuberculosis and as a adjunct therapy against *M. tuberculosis*. Moreover it will improve bone health and reduce HIV-1 disease progression. There is need for further supplementation studies in HIV-1 infected individuals to determine the optimal levels of vitamin D and to further

evaluate its safety *in vivo*. Also, more *in vitro* studies need to be done to investigate the mechanism by which vitamin D inhibits HIV-1 replication in the presence or absence of *M. tuberculosis*.

Chapter 7: The Influence of Macrophage Phenotype On The Differential Growth of *M. Tuberculosis* Strains and HIV-1 Replication During Co-infection

7.1 Literature review

Tuberculosis is often the first AIDS defining illness that manifests in HIV infected individuals in sub-Saharan Africa (Munyati *et al.*, 2005). Clinical progression in tuberculosis patients who are HIV infected is more rapid on first exposure or reactivation with short subclinical stage. Some patients who are HIV infected excrete the bacilli while they are not yet symptomatic (Wood *et al.*, 2007). There are many factors that affect disease outcome during *M. tuberculosis* and HIV co-infection such as host variability, HIV subtype and *M. tuberculosis* strain variation.

HIV and *M. tuberculosis* (HIV/TB) co-infection results in progressive disease as both pathogens accelerate the natural history of each other. In most cases individuals are exposed to *M. tuberculosis* infection first followed by infection with HIV later on in life. *M. tuberculosis* infection in HIV infected individuals is associated with increased HIV replication shown by high viral loads in co-infected individuals (Whalen *et al.*, 1995) and on the other hand HIV infection is thought to increase the growth of *M. tuberculosis* and its pathogenesis as shown by high mycobacterial sepsis in HIV infected individuals (Shafer *et al.*, 1989). The impact of *M. tuberculosis* strain variation in HIV infected individuals is not yet fully evaluated.

The successful sequencing of the *M. tuberculosis* genome enhanced the study of *M. tuberculosis* evolution and distinction of the strains (Cole *et al.*, 1998). During its evolution *M. tuberculosis* lost horizontal gene transfer capabilities (Alland *et al.*, 2003) and genetic variation occurs as a result of single nucleotide polymorphisms (SNPs) and large sequence polymorphism (LSPs), mutations in its DNA interspersed with repeats and tandem repeats, through deletions, duplications, inversions and insertions (Cole *et al.*, 1998).

It used gene duplication to diversify and acquire virulent genes like the early secretory antigen target-6 (ESAT-6) gene clusters, proline-glutamic acid (PE) and proline-proline-glutamic acid (PPE) genes (Nair *et al.*, 2011, Gey van Pittius *et al.*, 2006).

7.1.1 Clinical outcomes associated with *M. tuberculosis* strain variation.

Mycobacterium tuberculosis strains have differences in transmissibility, virulence, and immunogenicity that are determined by their genetics and environment. It is a diverse species and immune responses of the host to specific strains might influence the clinical outcome of the disease. Different *M. tuberculosis* strains activate different immunomodulatory pathways in the host with different cytokine networks and immune mediators being secreted (Nicol and Wilkinson, 2008).

M. tuberculosis strain variation can interact with host genotype and environment to influence disease outcome. Genetic variation of *M. tuberculosis* has been shown to result in ability of some strains to cause disease in different populations, rapid disease progression to active tuberculosis in recently exposed household contacts and ability to stimulate an effective immune response (Gagneux *et al.*, 2006, de Jong *et al.*, 2008, Rakotosamimanana *et al.*, 2010).

Different *M. tuberculosis* strains have unique virulence characteristics that influence disease outcome. The lineage 2, East Asian strains are associated with disease outbreaks, treatment failure, high virulence, multidrug resistance, co-infection with HIV, extra-pulmonary disease, suppressed pro-inflammatory immune responses and increased expression of macrophage deactivating cytokines IL-4 and IL-13 (Reed *et al.*, 2004, Caws *et al.*, 2006, Middelkoop *et al.*, 2009, Manca *et al.*, 2004). The virulence of Beijing strains is attributed to phenolic glycolipid (PGL) that modulates the pro-inflammatory response to *M. tuberculosis* (Hanekom *et al.*, 2011, Ordway *et al.*, 2007) although it is not the only determinant of virulence of Beijing strains (Sinsimer *et al.*, 2008).

On the other hand the virulence of the CDC1551 strain of lineage 4 is ascribed to its ability to stimulate a strong pro-inflammatory response by comparison to the HN878 strain of lineage 2, which suppress the pro-inflammatory response (Manca *et al.*, 2005, Manca *et al.*, 2001, Coscolla and Gagneux, 2010). In another study, CDC1551 was shown to induce a more pro-inflammatory response than the laboratory reference strain H37Rv and the Erdman strain even though the Edman strain is more virulent than the H37Rv and CDC1551 strain (Manca *et al.*, 1999). In addition Portevin and colleagues showed that lineage 1, 5 and 6 were associated with a higher pro-inflammatory cytokine response compared to lineages 2, 3 and 4 (Portevin *et al.*, 2011). Similarly clinical strains from lineage 2 have been shown to induce low pro-inflammatory cytokines IL-12p40 and TNF than lineage 4 clinical strains (Sarkar *et al.*, 2012) and H37Rv also infection of macrophages with these strains was associated with high necrosis and low apoptosis (Sohn *et al.*, 2009, Tanveer *et al.*, 2009).

Moreover *M. tuberculosis* strains have been shown to have differences in tissues they preferentially infect and ability to spread to other organs that is dependent on their virulence characteristics (Click *et al.*, 2012). In a study in Vietnam the Euro American lineage 4 was associated with pulmonary tuberculosis rather than extra pulmonary tuberculosis in particular meningeal tuberculosis (Caws *et al.*, 2008) whereas Lineage 3 CAS/ East African Indian and Indo-Oceanic of lineage 1 have been associated with extra pulmonary tuberculosis (Lari *et al.*, 2009, Click *et al.*, 2012). Therefore infection with different *M. tuberculosis* strains can potentially have a differential effect on the outcome of disease in HIV infected persons. HIV might modulate the host cells especially those co-infected in such a way that *M. tuberculosis* strains can induce differential HIV replication or differentially increase *M. tuberculosis* growth depending on the variable molecules they induce and secrete.

7.1.2 Immunopathogenesis of HIV and *M. tuberculosis* co-infection

Tuberculosis is the leading opportunistic infection during the course of HIV infection at any CD4 count and co-infection is associated with high mortality rates. The risk of tuberculosis increases as the degree of immunosuppression increases (Glynn *et al.*, 2008). Even after initiation of HAART and CD4 count recovery HIV infected individuals still have a higher risk of tuberculosis compared to HIV uninfected individuals (Badri *et al.*, 2002). This might be related to immune dysfunction of T cells and other components of the immune system caused by HIV (Lawn *et al.*, 2005). In HIV uninfected persons active tuberculosis results in formation of granulomas and progresses slowly where as in the HIV infected persons it forms diffuse lung infiltrates that occur fast and disease is progressive (Escombe *et al.*, 2008). The impact of the interaction between HIV and tuberculosis on tuberculosis infectiousness is not fully understood. There are conflicting results varying between increased or decreased infectiousness of HIV infected individuals, however a systematic review concluded that HIV had no effect on transmissibility of *M. tuberculosis* (Cruciani *et al.*, 2001).

The effect of HIV-1 subtypes and *M. tuberculosis* strain variation on the immune response to the HIV and *M. tuberculosis* during co-infection is not well defined. Different *M. tuberculosis* strains have been shown to stimulate host immune system differentially and stimulate the secretion of molecules that might have different effects on host immune system during HIV TB co-infection. There is need for more research to determine the effects of HIV subtypes and *M. tuberculosis* strain variation on the immune response to HIV and *M. tuberculosis* during co-infection.

7.1.2.1 The impact of the HIV-1 subtype on HIV/TB co-infection

There are increasing numbers of HIV-1 subtypes and circulating recombinant forms (CRF) that impact on the control of HIV infection by the immune system. Each HIV replication cycle results in generation of new HIV quasi species due to mutations caused by the poor fidelity of HIV-1 reverse transcriptase.

HIV strains and subtypes induce variable modulation of host immune response because of differences in gene sequences, which produce different proteins. These protein products stimulate the immune system in unique ways with activation of different transduction pathways that allow them to evade the host immune system (Nowak, 1992). HIV subtype D has been reported to be more virulent and results in high rate of disease progression and mortality compared to subtype A (Kiwanuka *et al.*, 2008, Baeten *et al.*, 2007). In addition subtype C, D and G were found to cause faster disease progression with development of AIDS within 5 years compared to subtype A and B (Kanki *et al.*, 1999). This might be related to subtype D high efficiency in shifting to producing CXCR4 tropic virions, syncytium formation and high rate of HIV replication (Tscherneing *et al.*, 1998, Kaleebu *et al.*, 2001). In terms of transmission HIV-1 subtype C is highly transmissible as it causes increased vaginal shedding compared the other HIV-1 subtypes (John-Stewart *et al.*, 2005).

Some HIV subtypes might be more responsive to molecules that are stimulated or released in response to different *M. tuberculosis* strains that can differentially stimulate HIV replication. This is because HIV-1 subtypes LTR respond to different molecules for example HIV-1 subtype E inhibits the transcription of the TNF gene in infected cells (Ranjbar *et al.*, 2006) therefore it is less responsive to TNF- α activation and T cell activation in absence of tat compared to subtype B LTR (Montano *et al.*, 1998, Lemieux *et al.*, 2004, Verhoef *et al.*, 1999). In addition HIV-1 subtype C is more transcriptionally active than subtype B and E, as its LTR is more responsive to NF κ B p65 (Montano *et al.*, 1997, Montano *et al.*, 1998).

7.1.2.2 The effects of HIV on tuberculosis

HIV infection has an impact on the natural history and progression of tuberculosis infection during co-infection. The presence of HIV results in defective granuloma formation as the granulomas during HIV infection lack the typical caseous necrosis instead they are composed of diffuse pulmonary infiltrates or lesions and necrosis (Diedrich and Flynn, 2011).

This results in poor control of *M. tuberculosis* growth and increased occurrence of disseminated miliary tuberculosis and subclinical active tuberculosis in HIV infected individuals due to reduced CD4+ T lymphocytes (Mtei *et al.*, 2005, Patel *et al.*, 2011).

HIV increases the risk of progression from latent tuberculosis to active tuberculosis and also causes rapid progression to active TB after exposure to new tuberculosis infection or relapse of disease in previously treated patients (Corbett *et al.*, 2003). This is related to the pathogenicity of HIV involving the depletion and dysfunction of CD4+ T cells (Antonucci *et al.*, 1995). HIV impairs the activation of T cells and immune responses to *M. tuberculosis* resulting in abnormal IFN- γ secretion (Sutherland *et al.*, 2006). In addition it reduces the number of macrophages and impairs their antimicrobial activities such as phagocytosis, maturation of the phagosome, intracellular killing (ROS), and cytokine secretion (Kedzierska and Crowe, 2002).

HIV infection also causes increased destruction of *M. tuberculosis* specific memory T lymphocytes (Wilkinson *et al.*, 2009). The depletion and dysregulation of CD4+ T lymphocytes due to HIV infection decreases the effectiveness of the protective immune response against *M. tuberculosis*. During primary HIV infection there is increased depletion of memory PPD specific IFN- γ secreting T cells as well as early secretory antigenic target-6 (ESAT-6) specific T cells which affect immunity to tuberculosis (Geldmacher *et al.*, 2008). In addition HIV proteins cause dysfunction of monocytes, monocyte derived macrophages and dendritic cells maturation and antimicrobial functions including the ability to respond to *M. tuberculosis* TLR ligands, phagocytosis, maturation of the phagosome, intracellular killing (ROS), and cytokine secretion and killing (Kedzierska and Crowe, 2002, Kalsdorf *et al.*, 2009, Collini *et al.*, 2010). Furthermore HIV/TB co-infection is associated with increased occurrence of drug resistant *M. tuberculosis* infection in HIV infected patients (Wells *et al.*, 2007). This might be caused by increased *M. tuberculosis* growth, malabsorption of drugs during HIV infection and the drug interactions of HAART and tuberculosis drugs.

7.1.2.3 The effects of *M. tuberculosis* on HIV

M. tuberculosis infection increases the viral replication in HIV infected individuals (Toossi, 2003). The lungs of individuals with active tuberculosis have high viral mRNA showing increased levels of HIV replication and transcription (Nakata *et al.*, 1997). In addition *M. tuberculosis* increases HIV replication in CD4⁺ T lymphocytes, monocyte derived macrophages and *ex vivo* in alveolar macrophages (Goletti *et al.*, 2004, Hoshino *et al.*, 2002, Zhang *et al.*, 1995, Mancino *et al.*, 1997). Infection with *M. tuberculosis* and HIV stimulates secretion of TNF- α that is involved in the restriction of *M. tuberculosis* growth whilst at the same time stimulates HIV replication (Kedzierska *et al.*, 2003).

Co-infection of dendritic cells with *M. tuberculosis* and HIV results in the suppression of the pro-inflammatory cytokine and up regulation of anti-inflammatory cytokine secretion causing defective activation of T cells by DC and allowing immune escape of HIV (Jiao *et al.*, 2002). *M. tuberculosis* stimulates the secretion of MIP-1 α , MIP-1 β and RANTES by macrophages that compete with HIV for CCR5 binding sites thereby inhibiting HIV infection (Kinter *et al.*, 1996, Saukkonen *et al.*, 2002). The manipulation of the cytokine networks by *M. tuberculosis* towards those that have an effect on HIV replication can result in increased disease progression during HIV/TB co-infection (Manca *et al.*, 1999, Reed *et al.*, 2004). *In vitro* studies, using PBMC have shown that different strains of *M. tuberculosis* induce differential HIV replication, with CDC1551 inducing increased HIV replication of HIV-1 subtypes B, C and E compared to HN878, which was attributed to increased generation of NF- κ B by the pro-inflammatory cytokines TNF, IL-6, and MCP-1 produced in response to CDC1551 strain (Ranjbar *et al.*, 2009b). However the impact for interaction of *M. tuberculosis* strains with HIV during co-infection of physiologically relevant macrophage phenotypes is unknown.

The mechanism and impact of interaction between *M. tuberculosis* strains and host pathogen interactions during HIV/TB co-infection are not yet fully understood. There is great need to investigate the mechanisms and sites at which HIV and *M. tuberculosis* influence each other.

In light of the study by Ranjbar et al 2009 which demonstrated differential stimulation of HIV replication by *M. tuberculosis* strains, there is need to examine the effect further at single cell level. Therefore this study investigates the hypothesis that different *M. tuberculosis* strains differentially stimulate of HIV replication in pro-inflammatory and anti-inflammatory macrophages. This study further examines the impact of HIV on the growth of *M. tuberculosis* strains to determine if the infecting strain affects outcome of infection during co-infection with HIV.

7.2 Results

7.2.1 Comparison of growth kinetics of *M. tuberculosis* strains

Three *M. tuberculosis* strains; H37Rv, LAM3 and HN878 were cultured in Middlebrook 7H9 liquid media supplemented with *albumin-dextrose-catalase* (ADC). The cultures were set up in triplicate for each strain, starting with a 1:100 inoculum dilution of the cultures at an OD of 0.01nm. Figure 7.1 shows the growth kinetics of the strains over 19 days. The growth curves of all the strains showed the typical sigmoid growth curve with the lag phase, exponential phase, stationary phase and death phase. All the strains had a short lag phase of 2 days before entering the exponential phase on the 3rd day. The exponential phase of the LAM3 strain was longer, as it went up to day 13, whereas that of H37Rv and LAM3 only went up to day 9 and 10 respectively. For all the curves, after the exponential phase, the typical horizontal line of the stationary phase was very short as the bacteria quickly entered the stationary phase.

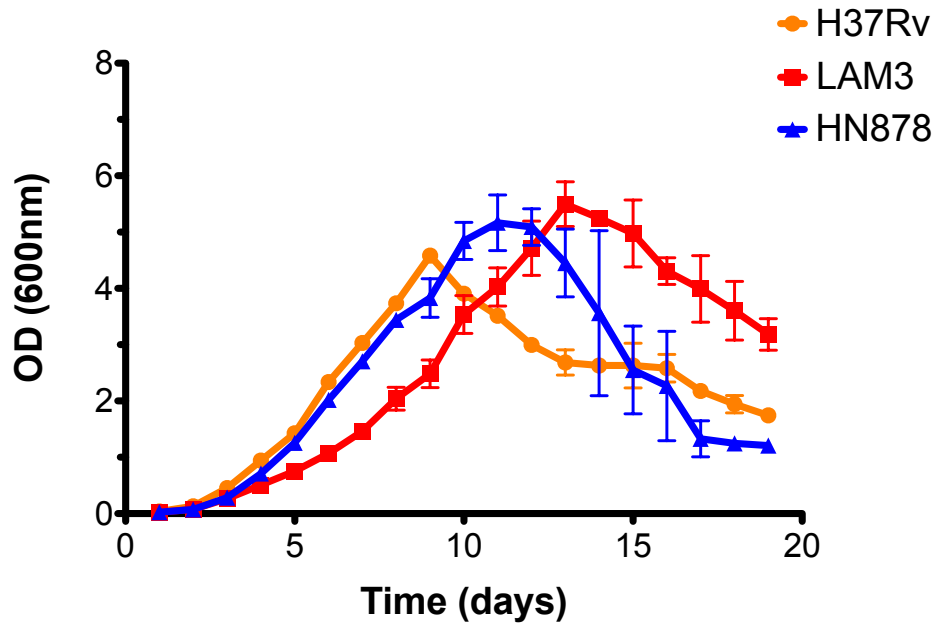


Figure 7.1 Growth kinetics of *M. tuberculosis* strains H37Rv, LAM3 and HN878 in Middlebrook 7H9 broth supplemented with ADC in triplicates. Errors bars represent mean \pm SD.

7.2.2 The effects of *M. tuberculosis* strain on HIV replication

In order to determine if HIV-1 p24 levels depend on *M. tuberculosis* strain variation and macrophage phenotype, GM-CSF macrophages (M ϕ -1) and M-CSF macrophages (M ϕ -2) were generated from monocytes matured from PBMC of 5 donors (Verreck *et al.*, 2004). The macrophages were infected with HIV-1 BAL at a MOI 1:3 for seven days followed by infection with *M. tuberculosis* strains H37Rv, LAM3 and HN878 at a MOI 1:1. HIV-1 p24 levels were measured at 4 hours, 24 hours, 96 hours and 168 hours post *M. tuberculosis* infection. The results of the p24 measurements are shown in Figure 7.2.

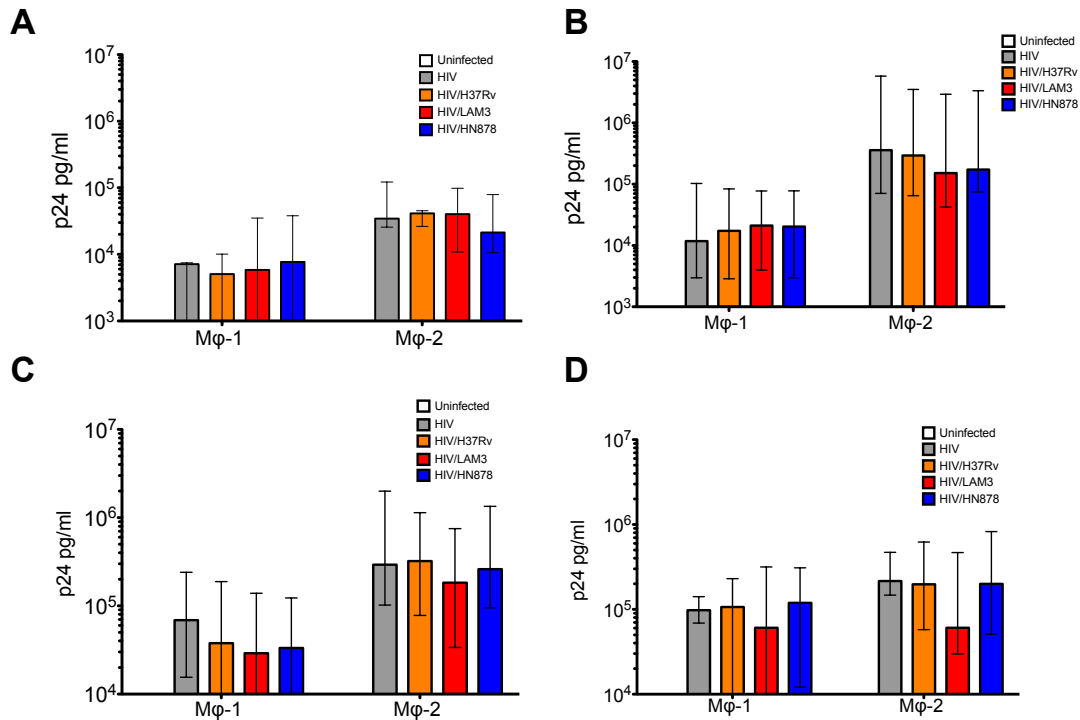


Figure 7.2 The effect of *M. tuberculosis* strain variation on HIV-1 p24 levels at specific time points (A) 4 hrs, (B) 24 hrs, (C) 96 hrs and (D) 168 hrs. Errors bars represent median and IQR (n=5).

A two-way analysis of variance (ANOVA) was conducted to evaluate the effects of infection with *M. tuberculosis* strains H37Rv, LAM3 and HN878 on p24 levels in Mφ1 and Mφ2 macrophages. *M. tuberculosis* strain variation had no effect on HIV replication. This was shown by no significant differences of p24 concentrations in response to infection with H37Rv, LAM3 or HN878 after 4 hrs ($p=0.90$), 24 hrs ($p=0.94$), 96 hrs ($p=0.79$) and 168 hrs ($p=0.92$) of infection. However, HIV-1 replication was significantly increased in Mφ2 compared to Mφ1, revealing an effect of macrophage phenotype, after 4 hrs ($p=0.002$), 24hrs ($p=0.05$), 96 hrs ($p=0.03$) and 168 hrs ($p=0.01$) (Figure 7.3). There was no interaction between macrophage phenotype and *M. tuberculosis* strain variation to influence HIV replication in Mφ1 and Mφ2 after 4 hrs ($p=0.58$), 24 hrs ($p=0.94$), 96 hrs ($p=0.88$) and 168 hrs ($p=0.81$).

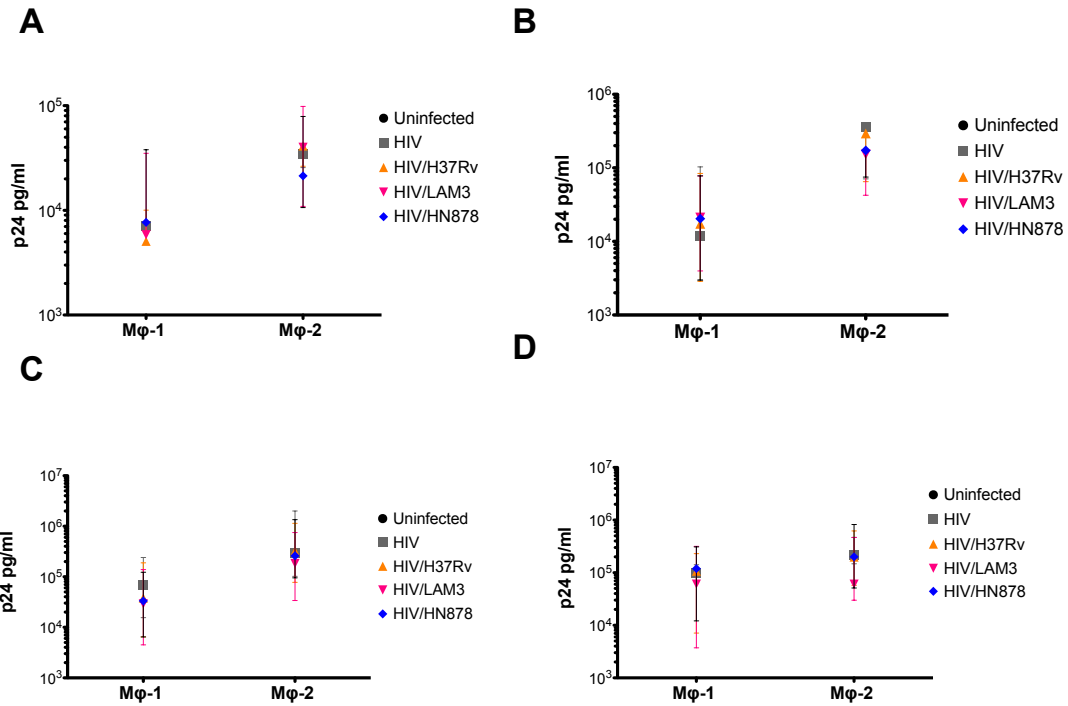


Figure 7.3 Comparison of HIV replication in Mφ1 and Mφ2 at specific time points (A) 4 hrs, (B) 24 hrs, (C) 96 hrs and (D) 168 hrs. Errors bars represent median and IQR, (n=5).

Post hoc pairwise comparisons with Bonferroni correction for multiple comparisons were performed to determine where the differences of macrophage phenotype were located. HIV replication was significantly increased in Mφ2 after 4 hrs of co-infection with H37Rv and LAM3 ($p < 0.05$ in both cases). Likewise, after 24 hrs, HIV replication was significantly increased in Mφ2 infected with HIV only ($p < 0.05$) and those that were co-infected with H37Rv and HN878 ($p < 0.05$ and $p < 0.001$ respectively). HIV replication during co-infection with H37Rv remained significantly increased in Mφ2 macrophages compared to Mφ1 after 96 hours and 168 hours of infection ($p < 0.05$ in both cases) (Figure 7.4).

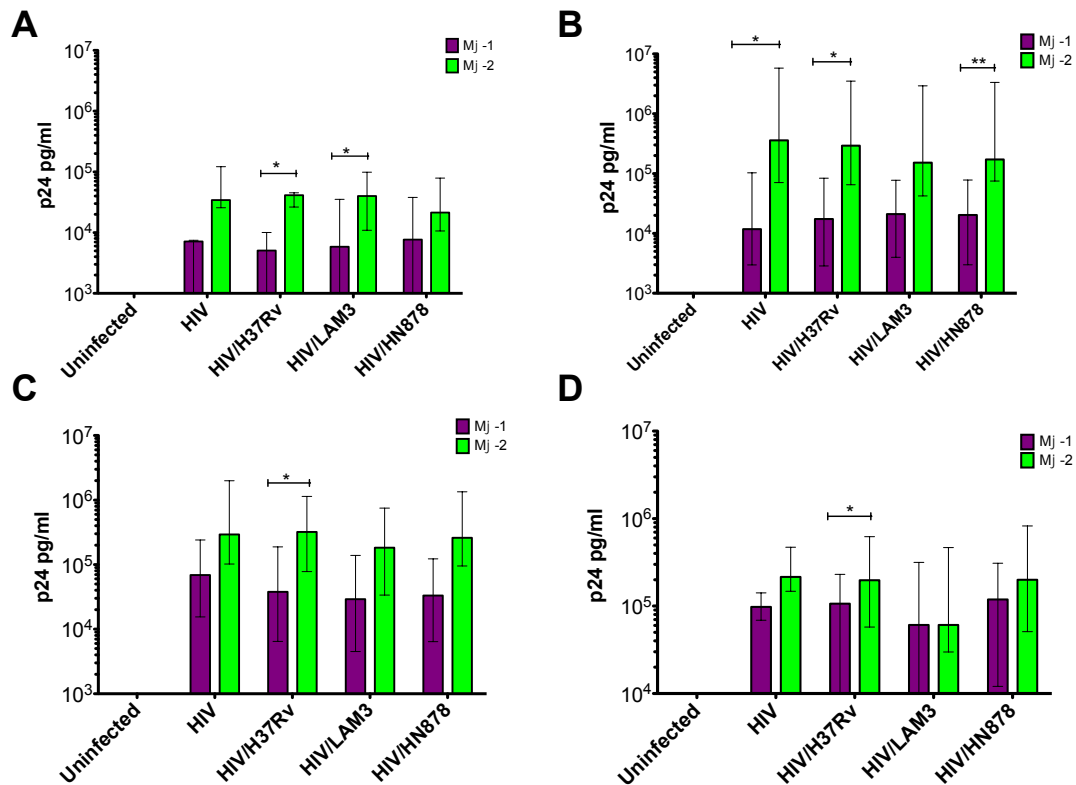


Figure 7.4 Effect of *M. tuberculosis* strains on HIV replication in Mφ-1 and Mφ-2 macrophages at specific time points (A) 4 hrs, (B) 24 hrs, (C) 96 hrs and (D) 168 hrs. Errors bars represent median and IQR (n=5). (* = p < 0.05).

Figure 7.5 shows the growth curve of HIV-1 in Mφ-1 and Mφ-2. There was a steep increase in p24 within the first 24 hours followed by a steady increase up to 168 hrs for Mφ-1. For Mφ-2, there was a steep increase of p24 levels in the first 24 hrs followed by a steady decrease that was greater in the HIV/LAM3 co-infected macrophages by 168 hrs. Overall HIV-p24 levels were not affected by *M. tuberculosis* strain type, but they were influenced by the macrophage phenotype, where increased HIV replication occurred in Mφ-2.

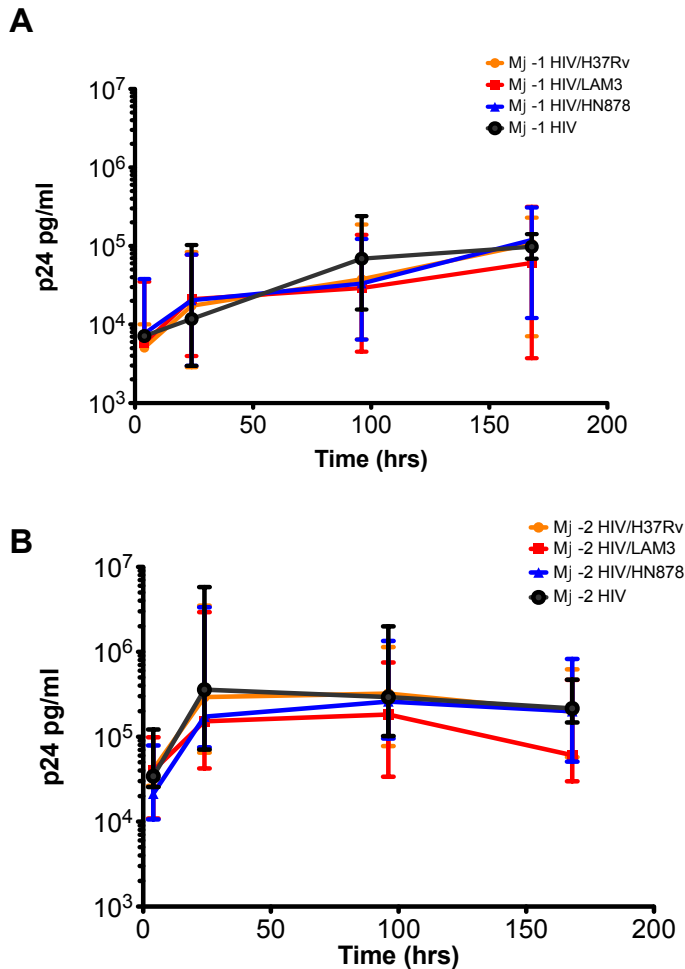


Figure 7.5 The growths curve of HIV in macrophages (A) represents M ϕ -1 and (B) M ϕ -2. Errors bars represent median and IQR (n=5).

7.2.3 The effect of HIV on *M. tuberculosis* strains growth.

7.2.3.1 *M. tuberculosis* strain uptake

The uptake of *M. tuberculosis* strains was determined by the number of CFU phagocytosed after 4 hrs of infection and the results were analysed by two way repeated measure ANOVA. Macrophage phenotype had no effect on the uptake of *M. tuberculosis* strains in HIV infected and HIV uninfected macrophages ($p=0.49$ and $p=0.42$ respectively). Instead *M. tuberculosis* strain variation had a significant effect, there was significant differences in the number of H37Rv, LAM3 and HN878 CFU phagocytosed by HIV infected ($p=0.03$) and HIV uninfected macrophages ($p=0.008$).

However there was no interaction between macrophage phenotype and *M. tuberculosis* strain variation to influence uptake of *M. tuberculosis* in HIV infected and HIV uninfected macrophages ($p=0.90$ and $p=0.70$ respectively). In addition the uptake of *M. tuberculosis* strains in M ϕ -1 and M ϕ -2 was independent of HIV infection ($p=0.88$ and $p=0.69$ respectively).

Post hoc pairwise analysis showed that there was a significant increased uptake of LAM3 strain compared to HN878 strain in HIV-uninfected M ϕ -1 ($p<0.05$) (Figure 7.6A). There was a trend towards increased uptake of the LAM3 strain compared to HN878 strain in HIV-infected M ϕ -1 and in both HIV-infected and uninfected M ϕ -2, though the pairwise comparisons were not statistically significant (Figure 7.6B).

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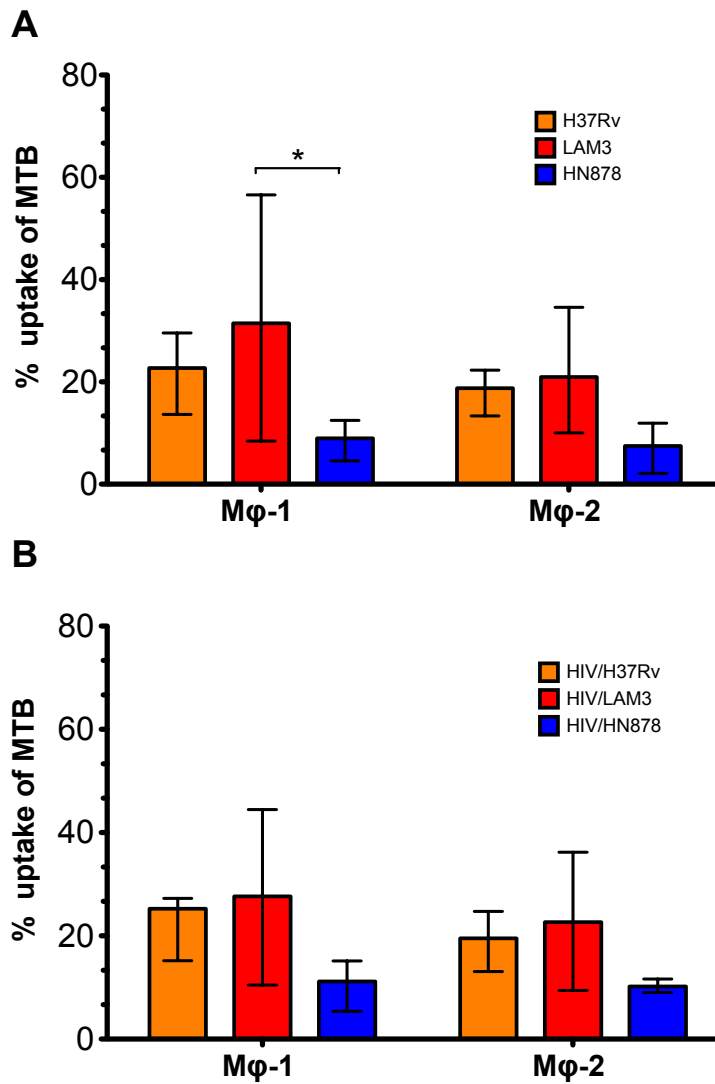


Figure 7.6 Two-way ANOVA of uptake of *M. tuberculosis* strains (A) HIV uninfected (B) HIV infected. Errors bars represent median and IQR (n=5), (* = p<0.05).

7.2.3.2 The effect of HIV on *M. tuberculosis* strain growth

To examine the effect of HIV infection and macrophage phenotype on growth of *M. tuberculosis* strains, two-way repeated measure ANOVA was used for analysis of the results. There was no significant difference in the growth of the *M. tuberculosis* strains between Mφ1 and Mφ2 after 24 hrs (p=0.99), 96 hrs (p=1) and 168 hrs (p=0.97) (Figure 7.7).

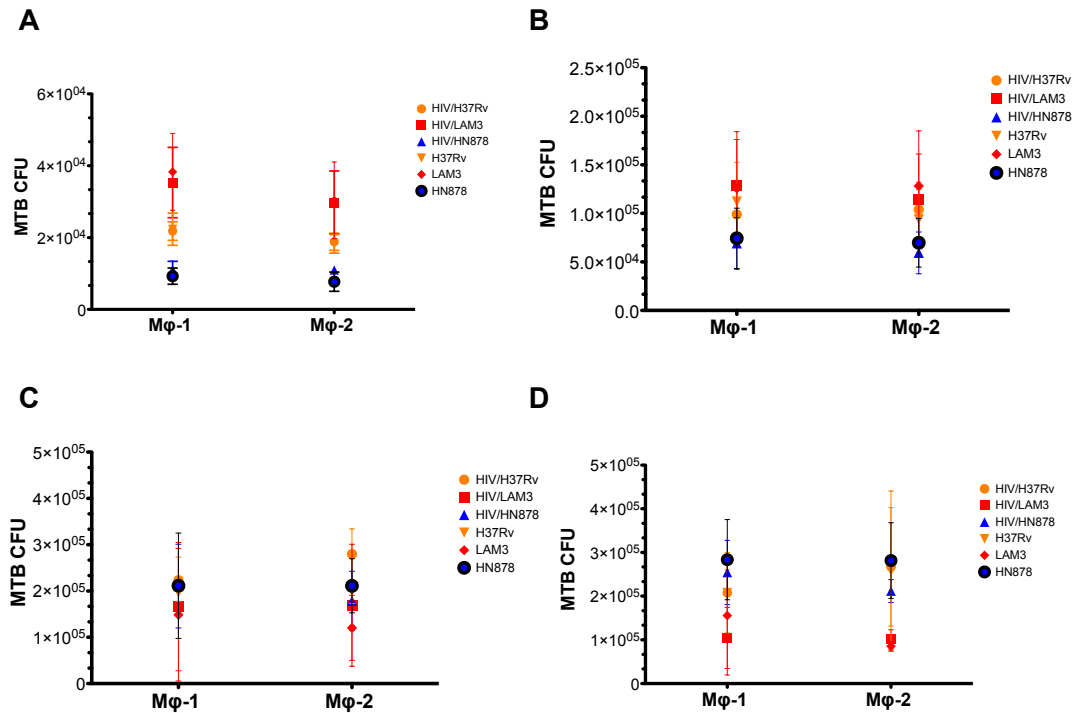


Figure 7.7 Variation in growth of the *M. tuberculosis* strains H37Rv, LAM3 and HN878 in Mφ1 and Mφ2 after (A) 4 hrs, (B) 24 hrs (C) 96hrs and (D) 168hrs of culture, (n=5). Errors bars represent median and IQR (n=5).

The effect of HIV infection on growth of *M. tuberculosis* strains was analysed. There was no significant difference in the growth of H37Rv, LAM3 and HN878 strains in the comparison of HIV infected and HIV uninfected Mφ1 after 24 hrs ($p=0.92$), 96 hrs ($p=0.80$) and 168 hrs ($p=0.59$) of infection (Figure 7.8 A-C). However *M. tuberculosis* strain variation had a significant effect on differences in *M. tuberculosis* CFU as there was a significantly increased growth of HN878 strain compared to LAM3 strain in both HIV uninfected ($p<0.05$) and HIV infected ($p<0.01$) Mφ1 after 168 hrs of infection (Figure 7.8C).

Similar to Mφ1 there was no significant difference in the growth of H37Rv, LAM3 and HN878 strains in the comparison between HIV uninfected and HIV infected Mφ2 after 24 hrs ($p=0.92$), 96 hrs ($p=0.41$) and 168 hrs ($p=0.56$) of infection (Figure 7.9A-C). There was significantly increased growth of H37Rv strain compared to LAM3 strain ($p<0.05$) and compared to HN878 ($p<0.008$) in HIV infected Mφ2 after 96 hrs (Figure 7.9B).

In addition, after 168 hrs of infection in HIV uninfected Mφ2, HN878 strain growth was significantly greater than that of LAM3 ($p < 0.01$), whereas in the HIV infected growth of H37Rv was significantly higher than that of LAM3 ($p < 0.05$, Figure 7.9C).

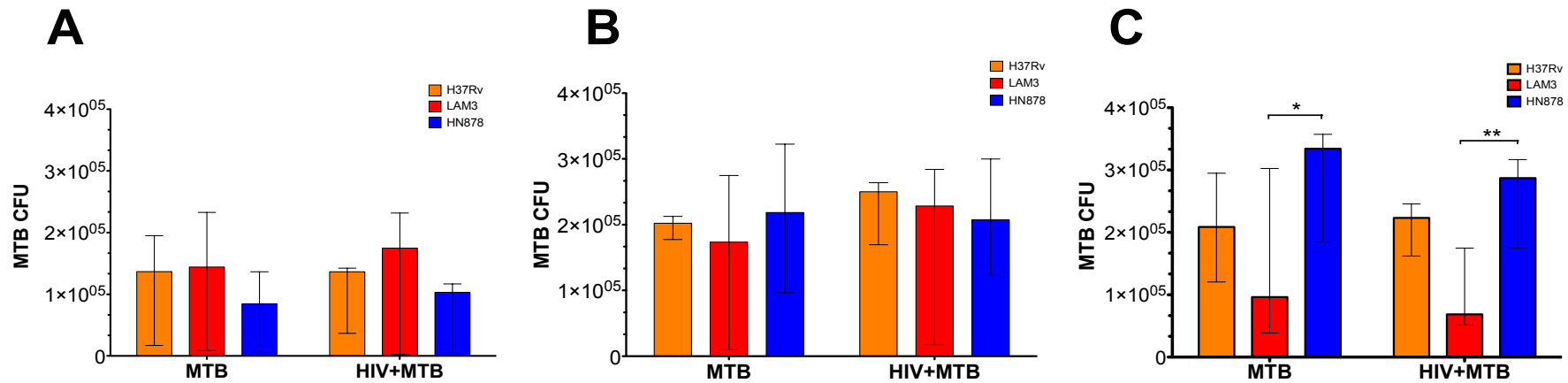


Figure 7.8 The growth of *M. tuberculosis* strains in Mφ1 stratified by HIV infection status after (A) 24 hrs, (B) 96 hrs and (C) 168 hrs. Errors bars represent median and IQR (n=5). (* = $p < 0.05$, ** = $P < 0.01$, *** = $p < 0.001$)

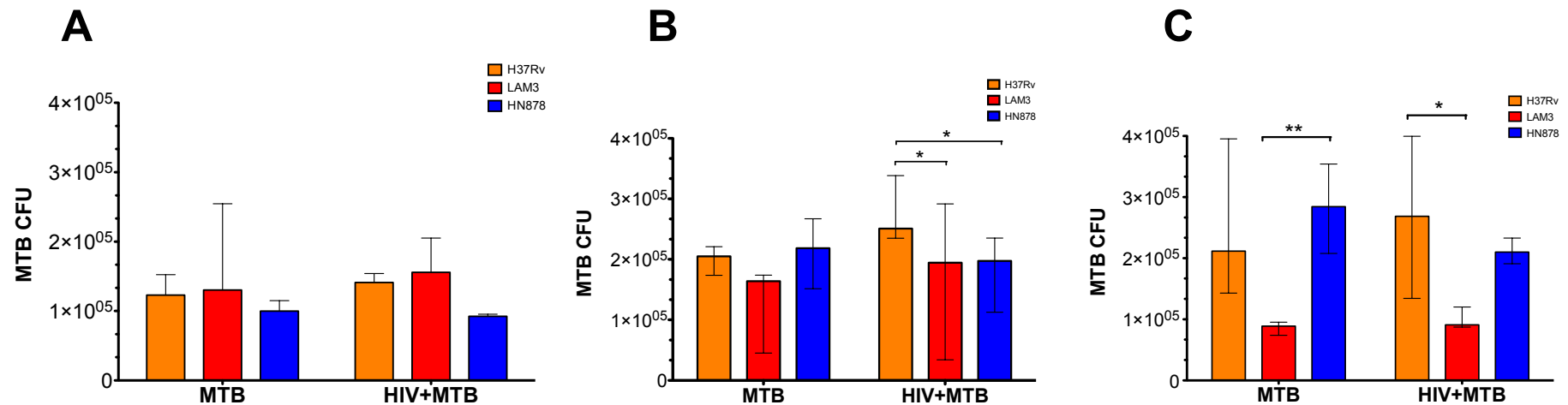


Figure 7.9 The growth of *M. tuberculosis* strains in Mφ2 stratified by HIV infection status after (A) 24 hrs, (B) 96 hrs and (C) 168 hrs. Errors bars represent median and IQR (n=5). (* = $p < 0.05$, ** = $P < 0.01$).

Figure 7.10 A and B shows the growth of H37RV, LAM3 and HN878 strains in M ϕ 1 and M ϕ 2 respectively. During the course of infection *M. tuberculosis* growth increases in the first 96 hrs and then starts to decrease steadily with a significant decrease in CFU for LAM3 in M ϕ 1 and M ϕ 2 after 168 hrs.

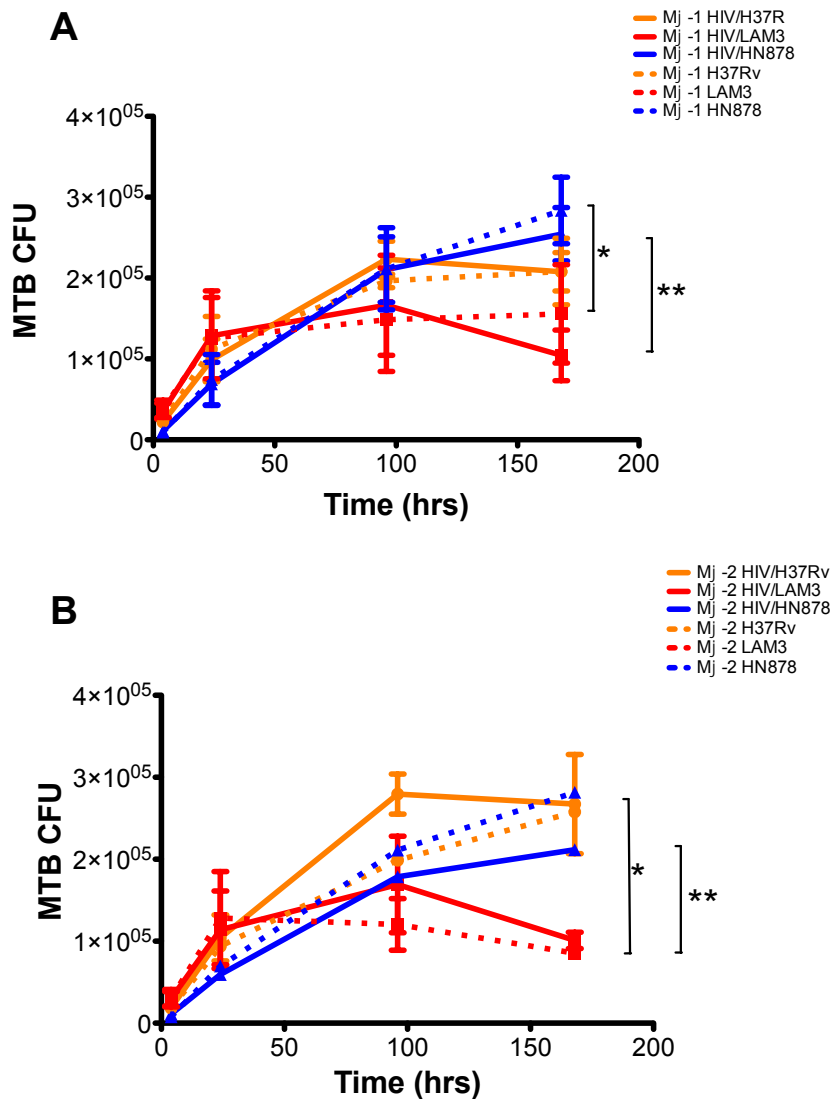


Figure 7.10 Growth curve of *M. tuberculosis* strains in (A) M ϕ 1 and (B) M ϕ 2. Errors bars represent median and IQR (n=5). (* = $p < 0.05$, ** = $P < 0.01$).

7.2 Discussion

The *M. tuberculosis* strains, H37Rv, LAM3 and HN878, with varying virulence had similar growth curves during *in-vitro* liquid culture, showing that the strains have comparable inherent capacities to replicate. My study found that these *M. tuberculosis* strains had no differential effect on HIV replication during co-infection of M ϕ -1 and M ϕ -2. On the other hand, neither HIV infection nor macrophage phenotype influenced the growth of the 3 strains. Nevertheless, there was significantly increased HIV replication in M ϕ -2 compared to M ϕ -1 macrophages and different *M. tuberculosis* strains had significantly different growth rates.

Increased HIV replication in lungs and other organ systems has been reported during co-infection with *M. tuberculosis*, (Nakata *et al.*, 1997, Toossi *et al.*, 2001, Matthews *et al.*, 2012). Despite that *in vivo* infection with *M. tuberculosis* results in increased HIV viral load and progression to AIDS (Whalen *et al.*, 1995), there are conflicting results on the *in vitro* effects of *M. tuberculosis* on HIV replication. Similar to the current results H37Rv had no effect on HIV replication (Meylan *et al.*, 1992). However in other studies *M. tuberculosis* strains H37Rv and CDC1551 increased HIV replication in M-CSF MDM (Pathak *et al.*, 2010, Ranjbar *et al.*, 2012), while in other cases it inhibited HIV replication (Goletti *et al.*, 2004). Use of unpurified HIV virions contaminated with host proteins and membranes from T cells that the virus was passaged through during preparation could result in increased HIV replication during co-infection in macrophages (Mikovits *et al.*, 1992).

In contrast to the current results *M. tuberculosis* strain variation induced differential HIV replication in PBMC with the CDC1551 strain stimulating the highest HIV replication compared to the HN878 strain (Ranjbar *et al.*, 2009b). The difference in results may be due to the use of different models of infection since differentiation of monocytes to macrophages is accompanied by the increased expression of the inhibitory 16kDa isoform of CCAAT enhancer binding protein β (C/EBP β), a transcriptional factor that inhibits activation of HIV replication (Weiden *et al.*, 2000, Honda *et al.*, 1998).

In fact, macrophages require cross-linking of their surface co-stimulatory molecules B7, CD40 and intercellular adhesion molecule -1 (ICAM-1) through contact with lymphocytes or neutrophils. This contact stimulates HIV replication through inhibition of the inhibitory 16kDa isoform of C/EBP β and increases the expression of the 37kDa isoform of C/EBP β that activates HIV replication by stimulation of NF-k β signalling (Hoshino *et al.*, 2002, Hoshino *et al.*, 2007). Therefore, in the PBMC model used by Ranjbar *et al.*, (2009) HIV replication was differentially stimulated by *M. tuberculosis* strains, possibly because of the contact of the lymphocytes with the mononuclear phagocytes that phagocytosed *M. tuberculosis*.

M. tuberculosis strain variation had a significant effect on the phagocytosis of *M. tuberculosis* however HIV infection had no effect on uptake of *M. tuberculosis*. Similar results have been reported elsewhere, phagocytosis of the H37Rv strain and *M. avium* has been shown to be the same in HIV-infected and uninfected macrophages (Meylan *et al.*, 1992, Elssner *et al.*, 2004). The increased phagocytosis of LAM3 compared to HN878 in HIV uninfected M ϕ 1 might be related to the mannosylation of lipoarabinomannan (ManLAM) since *M. tuberculosis* strains without ManLAM have decreased phagocytosis due to their inability to use the mannose receptor for entry into macrophages (Torrelles *et al.*, 2008).

The absence of effect of HIV on *M. tuberculosis* growth has also been observed by others (Kalsdorf *et al.*, 2009, Meylan *et al.*, 1992), although some *in vitro* studies have shown increased growth of *M. tuberculosis* during co-infection with HIV (Pathak *et al.*, 2010, Imperiali *et al.*, 2001). The different *M. tuberculosis* strains had different growth rates during the co-infection model. Previous studies have shown that different *M. tuberculosis* strain growth rates in macrophages and whole blood vary differentially depending on strain and virulence (Theus *et al.*, 2006, Wong *et al.*, 2007, Cheon *et al.*, 2002, Janulionis *et al.*, 2005). The increased growth of HN878 strain compared to LAM3 strain in both M ϕ 1 and M ϕ 2 after 168 hrs of infection might be due to the virulence of HN878 strain or ability of the macrophages to restrict growth of LAM3. However this conclusion is limited, because viability of the macrophages was not determined.

The decrease in LAM3 growth after 168 hrs might be caused by necrosis of LAM3 infected macrophages since LAM3 strains have been associated with increased apoptosis of polymorphonuclear cells (Romero *et al.*, 2012). Also continuous passaging of *M. tuberculosis* is associated with adaptation to *in vitro* growth and loss of some virulence factors (Domenech and Reed, 2009). In this study the LAM3 strain was passaged twice while the laboratory strains H37Rv and HN878 had been passaged a number of times. Variable growth rates of clinical *M. tuberculosis* strains compared to laboratory strains have been reported elsewhere (Li *et al.*, 2002).

Macrophage phenotype had no influence on growth of *M. tuberculosis* strains. Similarly, the growth of BCG was similar in M ϕ 1 and M ϕ 2 (Vogt and Nathan, 2011). However according to Akagawa, et al., M ϕ 2 are more effective in inhibition of *M. tuberculosis* growth than M ϕ -1, due to expression of the natural resistance-associated macrophage protein 1 (NRAMP1) in M ϕ 2, but not in M ϕ 1 (Akagawa *et al.*, 2006). In addition, in the mouse model Andrade, et al., report a significant decrease in H37Rv growth in pro-inflammatory macrophages (M1) compared to anti-inflammatory macrophages (M2) after 6 days of culture (Andrade *et al.*, 2012).

Activation of macrophages into a pro-inflammatory phenotype by IFN- γ and GM-CSF is important in the restriction of the growth of *M. tuberculosis* as they secrete pro-inflammatory cytokines (Herbst *et al.*, 2011, Fabri *et al.*, 2011, Denis and Ghadirian, 1990). M ϕ 2 are like alternatively activated macrophages and they secrete anti-inflammatory cytokines and are less microbicidal. The absence of effect of macrophage phenotype on *M. tuberculosis* growth might due to high plasticity of macrophages as infection of M ϕ 2 with HIV skews their phenotype towards M ϕ 1 (Chihara *et al.*, 2012) and virulent strains of *M. tuberculosis* modulate M ϕ 1 towards M ϕ 2 with reduced bactericidal activity (Ordway *et al.*, 2007, Andrade *et al.*, 2012).

The observed increased HIV replication in M ϕ 2 compared to M ϕ 1 has been corroborated by other studies in the absence of infection with *M. tuberculosis* (Kalter *et al.*, 1991, Komuro *et al.*, 2003, Matsuda *et al.*, 1995).

This is the first study to show that there is increased HIV replication in M ϕ 2 compared to M ϕ 1 during co-infection with *M. tuberculosis*. The increased HIV replication in M ϕ 2 compared to M ϕ 1 might be due to increased expression of stimulatory 37kDa isoform of C/EBP β and Hck in M ϕ 2 after HIV infection. M ϕ 2 have higher affinity for nef than M ϕ 1 and Hck binds nef which induces signal transduction pathways that increase NF- κ B signalling and HIV replication in M ϕ 2 (Chihara *et al.*, 2012, Komuro *et al.*, 2003, Saksela *et al.*, 1995). There is a need to elucidate the mechanism by which *M. tuberculosis* increases HIV replication during co-infection, as the above studies were carried out in the presence of HIV only.

The clinical implication of the increased HIV replication in M ϕ 2 is large. Monocytes in various tissues differentiate into macrophages in the presence of M-CSF and other immune-modulatory molecules. For example; osteoclasts develop in the presence of M-CSF and RANKL, while peritoneal macrophages develop in the presence of M-CSF (Asagiri and Takayanagi, 2007, Akagawa, 2002, Xu *et al.*, 2007). Therefore, increased HIV replication might occur in macrophages for long periods of time in tissues, some of which are immunologically protected since macrophages are long-lived cells. Further studies are needed in different types of tissue macrophages *ex vivo* and *in vitro* to verify this.

There is a great need for more investigations on the nature and mechanisms of the interaction between the host and co-infection with HIV and *M. tuberculosis* to identify genetic, immunological and molecular biomarkers that result in outcome of disease. The understanding of the pathogenesis and mechanisms by which the pathogens cause diseases is also important for identification of potential therapeutic targets.

Chapter 8: General Conclusion

The challenges of HIV/TB co-infection require development of novel interventions that are able to reduce the impact of TB on HIV infected individuals. Most suggested interventions include development of new drugs and that of pre and post TB vaccines. The development of novel immunotherapeutic interventions can also possibly improve the ability of HIV infected patients to resist active disease. Vitamin D is one of the immunotherapeutic agents, which can enhance the innate immune response against *M. tuberculosis* while at the same time modulating the pro-inflammatory immune response.

This study focused on the determination of the vitamin D status of Capetonians, with the particular aim of evaluating the relationship with active tuberculosis. In the absence of any studies of vitamin D and tuberculosis in this population, the cross-sectional design of the study allowed for the relatively fast collection of preliminary data. This provided a snapshot of the prevalence of vitamin D deficiency and related characteristics, and how they relate to ATB and LTBI status.

The results indicate for the first time the association of vitamin D deficiency with tuberculosis in HIV infected patients. Vitamin D deficiency might be one of the drivers which increases prevalence of TB among HIV infected patients. Active tuberculosis occurs throughout the course of HIV infection independent of CD4 count, which might reflect that other factors like vitamin D deficiency might play a role in increased susceptibility of HIV infected individuals to tuberculosis. The study provided the *in vivo* data of seasonal variation of vitamin D levels in Cape Town, South Africa that were reciprocal to the TB notifications. The predisposition of HIV infected individuals to development of active tuberculosis is influenced by both environmental and genetic factors.

The study of these factors that influence susceptibility of HIV infected individuals to tuberculosis could be useful in understanding immune response for development of vaccines as well as in biomarker discovery for identification of individuals with risk of developing active disease.

There is need for longitudinal studies, which includes individuals who do not have latent TB (TST and IGRA negative) to determine if there is a trend of decrease in vitamin D levels towards deficiency in the patients with active TB. The low vitamin D levels might result in suboptimal immunity to TB resulting in progression to active tuberculosis. Therefore a longitudinal study, which follows up on patients to determine the risk of development of TB in patients with vitamin D deficiency is needed to determine the mechanisms and role of vitamin D in immunity to tuberculosis.

There were no deleterious effects of vitamin D during infection of M ϕ 1 and M ϕ 2 with HIV and *M. tuberculosis*. In M ϕ 1 25(OH)D3 and 1,25(OH)₂D3 inhibited HIV replication while 1,25(OH)₂D3 inhibited Mtb growth during mono-infection. During co-infection 25(OH)D3 and 1,25(OH)₂D3 restricted HIV replication and *M. tuberculosis* growth. In M ϕ 2 only 25(OH)D3 inhibited HIV replication after 240 hrs of mono-infection and vitamin D molecules had no significant effect on HIV replication and *M. tuberculosis* growth during co-infection. These findings support that vitamin D supplementation can be a safe, cheap and effective way to enhance immunity against *M. tuberculosis* and HIV during co-infection. There is need for clinical trials to determine the dose and efficacy of vitamin D supplementation in HIV infected persons. Apart from the potential benefit of enhanced treatment of tuberculosis and prevention of latent TB activation, vitamin D supplementation in HIV infected persons can improve their bone health, as they are known to have low bone mineral density due to effects of HIV.

M. tuberculosis strains and HIV had no effect on each other's growth during co-infection in pro-inflammatory and anti-inflammatory macrophages. *M. tuberculosis* strain variation had no differential effect on HIV replication.

The three *M. tuberculosis* strains had different growth rates, which were independent of the macrophage phenotype or HIV co-infection. The study of the co-existence of HIV and *M. tuberculosis* strains in the macrophage is important for understanding the interaction of these pathogens with the host. HIV might modulate the cells in such a way that different strains of *M. tuberculosis* grow differentially which can have an impact on outcome of *M. tuberculosis* infection. The study of the impact of HIV on the growth of *M. tuberculosis* strains can shed light on the role of HIV in the rapid progression from latent tuberculosis to active tuberculosis in HIV infected persons since there is heterogeneity in rate of progression. More studies are needed to investigate the cytokine and gene expression changes during co-infection, which can allow identification of targets for treatment and biomarkers for diagnosis.

8.1 Presentations and publications

8.1.1 Publications

Adrian R Martineau, **Shepherd Nhamoyebonde**, Tolu Oni¹, Molebogeng X Rangaka, Suzaan Marais, Nonzwakazi Bangani, Relebohile Tsekela, Lizl Bashe, Virginia de Azevedo, Judy Caldwell, Timothy R. Venton, Peter M. Timms, Katalin A Wilkinson and Robert J Wilkinson 2011. Reciprocal seasonal variation in vitamin D status and tuberculosis notifications in Cape Town, South Africa. Proceedings of the National Academy of Sciences, 108, 19013-19017.

8.1.2 Poster presentation

Reciprocal seasonal variation in vitamin D status and tuberculosis notifications in Cape Town, South Africa.

Shepherd Nhamoyebonde¹, Adrian R Martineau^{2,3,4}, Tolu Oni^{1,4}, Molebogeng X Rangaka¹, Suzaan Marais¹, Nonzwakazi Bangani¹, Relebohile Tsekela¹, Lizl Bashe¹, Virginia de Azevedo⁵, Judy Caldwell⁵, Timothy R. Venton⁶, Peter M. Timms⁶, Katalin A Wilkinson^{1,2} and Robert J Wilkinson^{1,2,4}

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21st Century Challenges of Chronic Lung Disease, Cape Town, South Africa
November 2011

8.1.3 Poster abstracts

Macrophage phenotype influences cellular ability to control M. tuberculosis and HIV replication in response to vitamin D

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Keystone Host response in Tuberculosis, Vancouver , Canada March 2013.

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